Identification and characterization of miRNA-133b as a novel regulator of death receptor mediated apoptosis

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"The most exciting phrase to hear in science,
the one that heralds the most discoveries,
is not "Eureka!" (I found it!) but 'That's funny...'"
— Isaac Asimov

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Summary

MicroRNAs (miRNAs) represent a highly conserved family of endogenous non-protein-coding short RNA molecules which perform essential tasks in the regulation of eukaryotic cell homeostasis. During the past few years miRNAs have emerged as very potent controllers of both innate and adaptive immunity. Despite the profound consequences of this discovery for our understanding of immune response regulation hitherto virtually nothing is known about miRNA function during innate immunity to *Mycobacterium tuberculosis,* the causative agent of tuberculosis (TB).

Herein a miRNA expression profile of human THP1 macrophages infected with pathogenic *Mycobacterium tuberculosis* H37Rv or the vaccine strain *Mycobacterium bovis* BCG was generated. This led to the identification of miRNA-27a, 133b, 137, 145, 146a, 155, 339, 340 and let-7e as being differentially regulated during infection. These miRNAs were tested for their ability to regulate programmed cell death by using a well established and characterized experimental *ex-vivo* model of death receptor (DR)-induced apoptosis. Of all miRNAs tested, only miRNA-133b rendered apoptosis-resistant cells sensitive to tumor necrosis factor-alpha (TNFα)-activated cytotoxicity. Moreover, miRNA-133b treatment also resulted in exacerbated pro-apoptotic responses to *TNF*-related apoptosis-inducing ligand (TRAIL) or an activating antibody to CD95 (Fas/APO1). Comprehensive analysis, including microarray, pulsed stable isotope labeling by amino acids in cell culture (pSILAC) and *in-vitro* validation experiments, led to the discovery of the anti-apoptotic proteins Fas apoptosis inhibitory molecule (FAIM) and glutathione-*S*-transferase pi (GSTP1) as direct miRNA-133b targets. Moreover, underlining the pleiotropic and synergistic nature of miRNA activity, the expression of osteoprotegerin (OPG), a TRAIL decoy receptor, and fatty acid synthase (FASN), both genes with important anti-apoptotic and oncogenic features, could be further proven as miRNA-133b dependent. The results presented in this work represent the first known example of a single miRNA with the ability to influence all three major DR signaling pathways. Hence, miRNA-133b represents a very versatile pro-apoptotic molecule that achieves its goal by impairing direct regulators of DR ligand sensitivity (FAIM and OPG) as well as detoxifying (GSTP1) and survival metabolite-delivering enzymes (FASN). Since the expression of the strong pro-apoptotic miRNA-133b has been reported to be decreased in a wide array of different cancer types, this apoptosis and cell fate regulator seems to play a critical role in the cascade of events leading to cellular transformation and tumor generation.

Therefore, given the advantages provided by sequence-specific inhibition, miRNA-133b should be explored as a novel therapeutic target for anti-cancer treatment.

Expression of miRNA-133b in THP1 macrophages and HeLa cells was increased following innate immune activation by members of the Toll-like receptor (TLR) family. The strongest induction of miRNA-133b synthesis was observed after ligation of TLR3 with poly(I:C), suggesting that this miRNA is involved in the anti-viral response. MiRNA-133b enhanced the activity of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). This translated into increased levels of the pro-inflammatory interleukins 6 and 8 (IL6/8). Both the elevated activity of NF-κB and production of IL6 and 8 could be inhibited by co-transfection of miRNA-146a, a well known anti-inflammatory miRNA. These results identify miRNA-133b as one further member of a growing list of miRNAs with immunomodulatory functions. However, miRNA-133b differs from all other miRNAs described so far because it enhances inflammation, rather than dampening it. Since, exaggerated inflammation is a major cause of disease and tissue destruction, miRNA-133b represents a candidate target for molecular therapeutic intervention.

This work represents the first detailed characterization of miRNA-133b in the context of DR-mediated apoptosis and innate immunity. The molecular interactions dissected herein improve the understanding of the regulatory processes associated with tumorigenesis and the immune response.

Zusammenfassung

MicroRNAs (miRNAs) sind eine hoch konservierte Familie endogenener nicht-protein-kodierender kurzer RNA-Moleküle, die zentrale Aufgaben bei der Regulation der eukaryotischen Zellhomöostase erfüllen. MiRNAs wurden in den letzten Jahren als potente Regulatoren der angeborenen und adaptiven Immunität beschrieben. Trotz dieser Erkenntnisse blieb die Rolle dieser kurzen RNA Moleküle in Infektionen mit *Mycobacterium tuberculosis*, dem Erreger der Tuberkulose, bis dato weitgehend unerforscht.

Im Rahmen dieser Arbeit wurde ein miRNA-Expressionsprofil von menschlichen THP1 Makrophagen generiert, die mit dem pathogenen *Mycobacterium tuberculosis* H37Rv oder dem Impfstamm *Mycobacterium bovis* BCG infizierten waren. Dies ermöglichte die Identifizierung von miRNA-27a, 133b, 137, 145, 146a, 155, 339, 340 und let-7e als differenziell regulierte miRNAs bei der Infektion. Diese wurden anhand eines gut etablierten und charakterisierten *ex-vivo*-Modells von Todesrezeptor-induzierter Apoptose auf ihre Fähigkeit untersucht, das kontrollierte Zelltodprogramm zu beeinflussen. Von allen geprüften miRNAs führte nur miRNA-133b dazu, dass Zellen, die unter gewöhnlichen Umständen apoptoseresistent sein sollten, nun empfindlich gegen Tumornekrosefaktor-α (TNFα) induzierte Zytotoxizität wurden. Darüber hinaus verursachte die Behandlung der Zellen mit miRNA-133b auch verstärkte pro-apoptotische Antworten auf TNF-related apoptosis-inducing ligand (TRAIL) oder einen aktivierenden Antikörper gegen CD95 (Fas/APO1). Eine umfassende Studie bestehend aus RNA-Expressionsanalyse, dem sogenannten pulsed stable isotope labeling by amino acids in cell culture (pSILAC)- und *In-vitro*-Validierungsexperimenten führte zur Identifizierung der anti-apoptotischen Proteine Fas apoptosis inhibitory molecule (FAIM) und glutathione-S-transferase pi (GSTP1) als direkte Zielgene für miRNA-133b. Desweiteren zeigte sich die Expression von Osteoprotegerin (OPG), einem TRAIL- Köder-Rezeptor, und Fettsäuresynthase (FASN), zwei Gene mit wichtigen anti-apoptotischen und tumorerzeugenden Funktionen, als miRNA-133b abhängig. Dies unterstrich die pleiotrope und synergistische Art der pro-apoptotischen Aktivität dieser miRNA. Diese Ergebnisse bilden das erste bekannte Beispiel einer einzelnen miRNA mit der Fähigkeit, die drei wichtigsten Todesrezpetorsignalwege zu beeinflussen. Somit stellt miRNA-133b ein sehr vielseitiges pro-apoptotisches Molekül dar, das seine Wirkung durch die gezielte Beeinträchtigung von Apoptose-Empfindlichkeitsregulatoren (FAIM und OPG) sowie entgiftenden (GSTP1) und Metabolit-bereitstellenden Enzymen (FASN) erreicht. Die

Stummschaltung der pro-apoptotischen Wirkung von miRNA-133b scheint eine wichtige Rolle bei der Tumorentstehung zu spielen, weil die Expression der miRNA in einer Vielzahl unterschiedlicher Krebsarten herunterreguliert wird. Aus diesem Grunde könnte die gezielte Aktivierung von miRNA-133b oder eine ektopische Expressionssteigerung einen interessanten neuartigen Therapieansatz in der Krebsbekämpfung darstellen.

Die Expression von miRNA-133b wurde in THP1 Makrophagen und HeLa-Zellen durch Mitglieder der Toll-like Rezeptor (TLR)-Familie aktiviert. Die stärkste Induktion von miRNA-133b wurde nach Stimulation von TLR3 mit Poly(I:C) erreicht. Dies deutet daraufhin, dass diese miRNA an der anti-viralen Immunantwort beteiligt ist. MiRNA-133b Transfektion führte zu einer verstärkten Aktivierung des Transkriptionsfaktors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Dies resultierte in erhöhten Mengen an pro-inflammatorischen Interleukinen 6 und 8 (IL6/8). Sowohl die erhöhte NF-κB-Aktivität als auch die stärkere Produktion von IL6/8 konnte durch Co-Transfektion von miRNA-146a, einer entzündungshemmenden miRNA, blockiert werden. Diese Ergebnisse identifizieren miRNA-133b als ein weiteres Mitglied einer wachsenden Anzahl von miRNAs mit immunmodulatorischen Funktionen. MiRNA-133b unterscheidet sich allerdings von den anderen miRNAs darin, dass sie den Entzündungsprozess fördert anstatt ihn zu dämpfen. Da unkontrollierte Entzündungen eine der Hauptursachen von Krankheit und Gewebezerstörung sind, bietet sich miRNA-133b als eine interessante potenzielle Angriffsstelle für molekulare therapeutische Intervention an.

Diese Arbeit stellt die erste detaillierte Charakterisierung von miRNA-133b im Zusammenhang der Todesrezeptor-vermittelten Apoptose und der angeborenen Immunität dar. Die erforschten molekularen Wechselwirkungen ergänzen und bereichern das Verständnis über die regulatorischen molekularen Mechanismen, die mit der Tumorentstehung und Entzündung verbunden sind.

Abreviations

ΔΨm	mitochondrial transmembrane potential
% (v/v)	volume percent per volume
% (w/v)	mass percent per volume
µ	micro (10^{-6})
bp	base pair
BSA	bovine serum albumine
ctrl.	control
DcR	decoy receptor
DD	death domain
ddH2O	double distilled water
DISC	death inducing signaling complex
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DR	death receptor
DTT	dithiothreitol
EDTA	ethylendiamintetraacetic acid
ELISA	enzyme linked immunosorbent assay
EtBr	ethidium bromide
FACS	fluorescent-activated cell scanner
FAIM	Fas apoptosis inhibitory molecule
FasL	Fas ligand
FASN	fatty acid synthase
FCS	fetal calf serum
FLICA	fluorescent-labeled inhibitors of caspases
fw	forward
GSTP	glutathione S-transferase pi 1
h	human
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
hrs.	hours
IFN	interferon
kDa	kilodalton
kb	kilobases
LPS	lipopolysaccharide
mfe	mean free energy
min.	minute
miRNA	microRNA

ml	mililiter
MMP	mitochondrial membrane permeabilization
MOI	multiplicity of infection
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n	Nano (10^{-9})
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
nm	nanometer ($10^{-9)}$
OD	optical density
OPG	osteoprotegerin
PAGE	polyacrylamide gel electrophoresis
PARP	poly [ADP-ribose] polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
PS	phosphatidylserine
pSILAC	pulsed stable isotope labeling by amino acids in cell culture
PVDF	polyvinylidene fluoride
r	recombinant
RISC	RNA induced signaling complex
re	reverse
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulfate
TB	tuberculosis
TEMED	*N*,*N*,*N*′,*N*′-tetramethyl-ethane-1,2-diamine
TLR	Toll like receptor
TNFR	tumor necrosis factor receptor
TNFα	tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
Tris	tris(hydroxymethyl)aminomethane
TUNEL	dUTP Western blot nick end labeling
WB	

1. Introduction

1.1 MicroRNAs

The controlled expression of genes is an essential feature of living eukaryotic cells controlled by several different mechanisms. MicroRNAs (miRNAs) are an abundant class of newly identified and highly conserved endogenous non-protein-coding short (~22 nt) RNA molecules that play an essential role in the regulation of cellular homeostasis (Bartel, 2004). miRNAs have been shown to play cardinal roles in biological processes including tissue development, stem cell differentiation, innate and adaptive immune responses (Hou et al, 2009; Houbaviy et al, 2003; Judson et al, 2009; Lee et al, 1993; O'Connell et al, 2010; Taganov et al, 2007; Wang et al, 2008). Currently, the best characterized function of miRNAs is the fine tuning of gene activity at the post-transcriptional level by impairing gene expression.

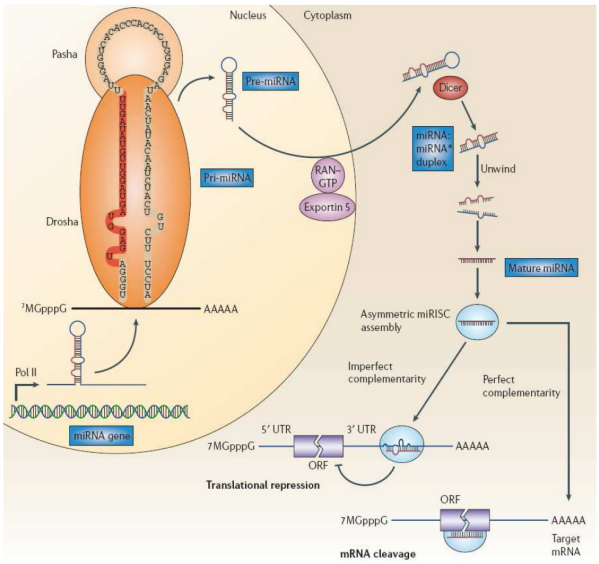
1.1.1 History

The history of miRNAs started in the early years of last century´s decade when two independent groups discovered that a small RNA molecule named *lin-4* was involved in *Caenorhabditis elegans* development by regulating the expression of *lin-14,* a gene responsible for temporal pattern formation (Ambros & Horvitz, 1987; Lee et al, 1993; Wightman et al, 1993). Although these reports did not include a precise mechanistic description of the studied phenomenon, two seminal observations for future miRNA research were made: *lin-4* is not protein-coding and it contains antisense sequences complementary to a repeated motif in the 3´-untranslated region (3´-UTR) of the *lin-14* mRNA. Despite these relevant results, it took seven years until *let-7* was discovered as a small regulatory RNA highly conserved in a wide range of animal species and real interest in these novel class of molecules started to grow (Pasquinelli et al, 2000; Reinhart et al, 2000). This was evidenced one year later, in 2001, by publication of three groundbreaking reports on the identification of a large number of short RNAs similar to *lin-4* and *let-7* in different animal species. This was the first time that the term miRNA was coined to denominate this novel class of expression regulators (Lagos-Quintana et al, 2001; Lau et al, 2001; Lee & Ambros, 2001). Ever since, miRNAs have become subject of very intensive research. As of September 2009 more than 10 000 miRNA sequences had been deposited in repository genomic databases and it was

estimated *in-silico* that miRNAs may represent up to 5% of all genes of an organism (Lim et al, 2003a; Lim et al, 2003b). Moreover, 30% of all human protein-coding genes are predicted to be regulated post-transcriptionally by miRNAs (Lewis et al, 2005).

1.1.2 miRNA biogenesis

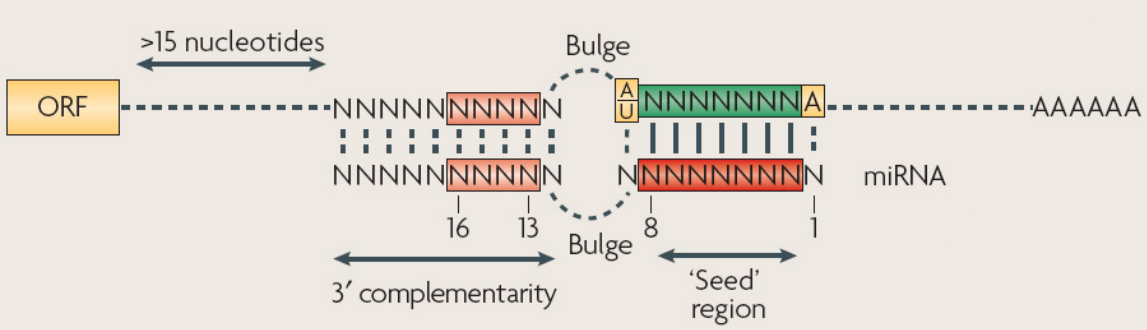
Generation of functional miRNAs is a complex multi-enzyme process leading from long precursor molecules into ~22 nt long biologically active RNA molecules. MicroRNA genes are generally transcribed in the nucleus by RNA polymerase II (Pol II) in large primary miRNA transcripts (pri-miRNA) that undergo normal further processing *i.e.* 5´-capping, 3´-polyadenylation or even splicing (Cai et al, 2004). These RNA molecules form specific hairpin-shaped stem-loop secondary structures and enter a multi-enzyme complex known as a microprocessor to be modified by the RNAse III enzyme Drosha and its co-factor, Pasha. This leads to the formation of a ~70 nt precursor miRNAs (pre-miRNA) with a 5´-phosphate and a 3´- 2 nt long overhang (Denli et al, 2004). Export of pre-miRNAs to the cytoplasm is performed by ras-related nuclear protein (RAN-GTP) and exportin 5 (XPO5) (Bohnsack et al, 2004). Cytoplasmic pre-miRNAs are further processed by another RNAse III enzyme termed Dicer to generate a transient ~22 nt long double stranded miRNA (Hutvagner et al, 2001). This duplex is unwounded by helicases into two single strands, one of which is then loaded into the miRNA-associated multiprotein RNA-induced silencing complex (miRISC) which includes the Argonaute proteins and induces gene silencing (Schwarz et al, 2003) (Scheme 1).



Scheme 1 Molecular mechanisms of miRNA biogenesis and function (Esquela-Kerscher & Slack, 2006)

1.1.3 Mechanisms of miRNA-mediated gene regulation

In most cases miRNAs interact with their targets by base pairing to sequences located within the 3´-UTR of the mRNA. However, it has also been reported that miRNAs can exert their regulatory function by targeting sequences located in the 5´-UTR or coding sequence (cds) (Duursma et al, 2008; Forman et al, 2008; Lewis et al, 2005; Spizzo et al; Zhou et al, 2009). Currently, three features important for miRNA-target interactions have been identified bioinformatically and experimentally. First, nucleation of miRNA:mRNA interactions by contiguous base pairing of miRNA nucleotides 2 to 8 (also known as “seed” region) (Doench & Sharp, 2004). Second, bulges or mismatches must be present in the central region of the miRNA-mRNA duplex (Watanabe et al, 2006). The third rule is that there must be good complementarity to the miRNA 3´ half to stabilize the interaction (Brennecke et al, 2005) (Scheme 2). Other variables that favor the efficiency of miRNA-mediated silencing are an AU-rich region and a position close to the poly(A) tail or the termination codon (Grimson et al, 2007; Nielsen et al, 2007) (Scheme 2). Finally, in animal transcripts imperfectly paired target sites for the same miRNA often occur multiple times within the same mRNA. This multiplicity of binding regions correlates directly to the magnitude of gene expression repression (Doench & Sharp, 2004).



Scheme 2. Molecular principles and rules governing miRNA-mRNA interactions (Filipowicz et al, 2008)

Depending on the degree of complementarity between the miRNA and its target, it can direct RISC to down-regulate gene expression by either of two post-transcriptional mechanisms: translational arrest or mRNA cleavage (Hutvagner & Zamore, 2002). Translational arrest is mediated by molecular mechanisms taking place at two different stages during protein biosynthesis (Eulalio et al, 2008):

- Initiation mechanisms: miRNAs and RISC can interfere with very early steps of mRNA translation. For instance, Argonaute proteins compete with initiation factors for binding to the cap structure of mRNAs (Kiriakidou et al, 2007) and, they can recruit inhibitory factors that prevent the joining of the 40 and 60S ribosomal units (Chendrimada et al, 2007).

- Post-initiation mechanisms: miRNAs can block translation elongation by promoting premature ribosomal dissociation ("ribosome drop-off") (Maroney et al, 2006; Petersen et al, 2006). Moreover, a third model proposes that the nascent polypeptide chain might be degraded co-translationally (Nottrott et al, 2006).

In eukaryotes, miRNA-mediated mRNA degradation can follow two pathways, both of which are initiated by gradual shortening of the mRNA poly(A) tail (Filipowicz et al, 2008). This can lead to further 3′→5′decay, which is catalyzed by the exosome or, to removal of the cap followed by 5′→3′ degradation (Behm-Ansmant et al, 2006; Giraldez et al, 2006).

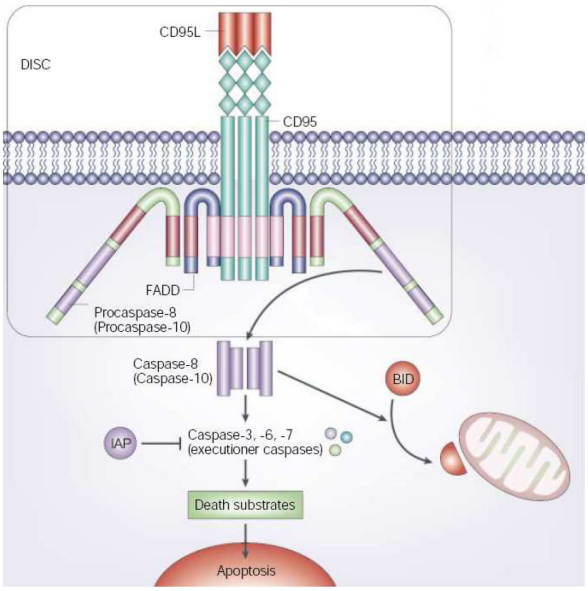
1.2 Apoptosis

Apoptosis is a form of active programmed cell death (PCD) inherently determined and strictly regulated, that plays an important role for a plethora of processes in multicellular organisms (Kerr et al, 1972). Apoptosis occurs normally during development, aging and as a homeostatic mechanism to maintain equilibrium between cell populations in tissues and organs. It also occurs as a defense mechanism such as in immune reactions or when cells are severely damaged by disease or cytotoxic agents. Cells undergoing apoptosis are characterized by well defined morphological and biochemical changes such as rounding-up of the cell, retraction of pseudopodes, reduction of cellular volume, classically little or no ultrastructural modifications of cytoplasmic organelles, caspase activation, decay of mitochondrial transmembrane potential ($\Delta\Psi m$), activation of pro-apoptotic molecules, chromatin condensation, mitochondrial membrane permeabilization (MMP), nuclear fragmentation, and membrane blebbing (Kroemer et al, 2009). Another biochemical feature is the expression of cell surface markers such as phosphatidylserine (PS) that result in the early phagocytic recognition of apoptotic cells by adjacent phagocytes, permitting quick clearance and minimal compromise to the surrounding tissue (Bratton et al, 1997; Elmore, 2007). This process of controlled cellular suicide can be triggered by extracellular and intracellular stimuli, both of which result in the activation of specific, yet partially overlapping molecular mechanisms.

1.2.1 Extrinsic apoptotic pathway

The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These encompass death receptors (DRs) which represent a group of extracellular membrane-bound molecules responsible for sensing and transducing exogenously derived pro-apoptotic signals. DRs, including the tumor necrosis factor receptor 1 and 2 (TNFR1/2), Fas/APO1 (CD95) and TNF-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5, belong to the TNF superfamily and share similar cysteine-rich extracellular domains and a common structurally conserved 80 amino-acid long cytoplasmic death domain (DD) (Aggarwal, 2003; Ashkenazi & Dixit, 1998). Upon cognate ligand binding, DRs oligomerize via their DD giving rise to a scaffold for the recruitment of several adaptor and signaling molecules (Grimm et al, 1996; Hsu et al, 1995). At this death inducing signaling complex (DISC), initiator caspases such as caspase 8 or 10 (CASP8/10) get

activated by means of autocatalytic cleavage (Kischkel et al, 1995). Once triggered, initiator caspases launch the execution phase of the death signal by processing effector caspases, like CASP3, 6 or 7, to their active forms (Scheme 3).



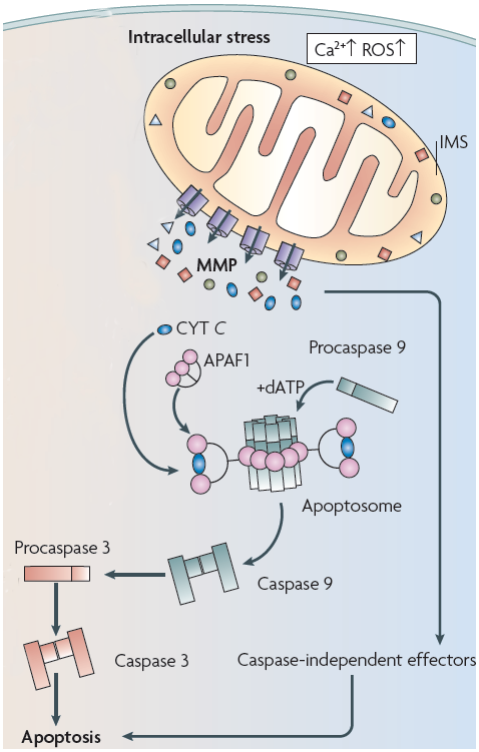
Scheme 3. Apoptosis signaling through death receptors as exemplified by Fas/FasL signaling system. Modified after (Igney & Krammer, 2002)

As a consequence of this released proteolytic activity, key intracellular factors, also known as death substrates, are degraded. Cleavage of nuclear lamins is involved in chromatin condensation and nuclear shrinkage (Rao et al, 1996). Proteolysis of the inhibitor of caspase activated deoxyribonuclease (ICAD) causes the release of the endonuclease, which travels to the nucleus to fragment DNA (Enari et al, 1998). Cleavage of cytoskeletal proteins such as actin, plectin, Rho kinase 1 (ROCK1) and gelsolin leads to cell fragmentation, blebbing and the formation of apoptotic bodies (Chen et al, 1996; Kothakota et al, 1997; Sebbagh et al, 2001; Stegh et al, 2000). After execution of the apoptotic program the remains of the dying cell are engulfed by phagocytes (Savill & Fadok, 2000).

1.2.2 Intrinsic apoptotic pathway

Intrinsic apoptosis is triggered by several conditions of intracellular stress, such as reactive oxygen species, DNA damage, hypoxia and Ca^{2+} overload. All these stimuli disembogue in changes of the mitochondrial membrane that lead to the opening of the

mitochondrial permeability transition pore (MPT). Consequently, ΔΨm is lost and proteins from the mitochondrial intermembrane space (IMS) are released into the cytosol (Scheme 4), where they promote cell death by multiple mechanisms (Kroemer et al, 2007).



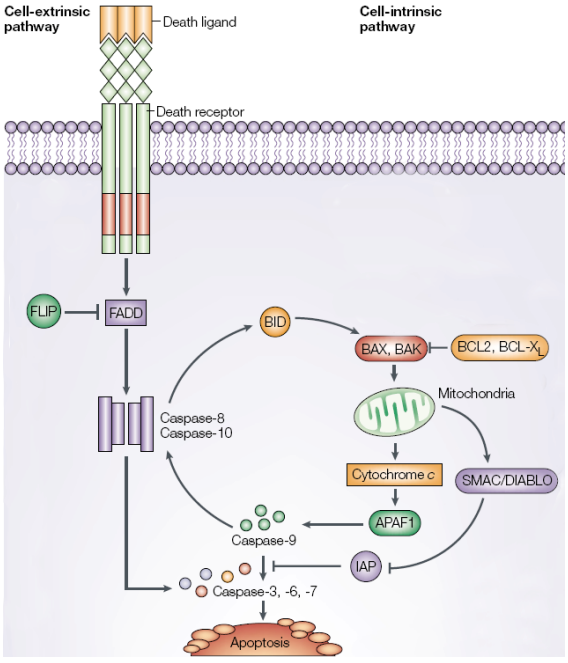
Scheme 4. Mitochondrial (intrinsic) apoptosis pathway. Modified after (Galluzzi et al, 2009).

Released proteins include cytochrome c (CYTC), second mitochondria-derived activator of caspases/direct inhibitors of apoptosis proteins (IAP)-associated binding protein with low pI (SMAC/DIABLO), the serine protease high temperature requirement protein A2 (HtrA2/OMI), apoptosis-inducing factor (AIF), endonuclease G (EndoG) and caspase activated deoxyribonuclease (CAD) (Du et al, 2000; Enari et al, 1998; Li et al, 2001; Liu et al, 1996a; Susin et al, 1996; van Loo et al, 2002). CYTC oligomerizes with apoptotic peptidase activating factor 1 (APAF1), dATP and CASP9 forming the apoptosome. In this complex caspase-9 is activated by oligomerization and can then process other caspases, such as caspase-3, thus committing the cell to suicide (Zou et al, 1999). SMAC/DIABLO and HtrA2/OMI are reported to promote apoptosis by antagonizing the activity of members of the IAP family (van Loo et al, 2002). Cytoplasmic AIF, EndoG and CAD contribute to DNA fragmentation and subsequent chromosomal condensation (Enari et al, 1998; Joza et al, 2001; Li et al, 2001). MPT independent MMP is regulated by B-cell lymphoma 2 (BCL2) family members, which are characterized by BCL2 homology (BH) domains (Wang & Youle, 2009). The Bcl-2 family can be subdivided into anti-apoptotic members such as BCL2, BCL-

X_L and BCL-w and pro-apoptotic species such as BCL2-associated X protein (BAX), BCL2-antagonist/killer 1 (BAK), BCL2-associated agonist of cell death (BAD), BH3 interacting domain death agonist (BID) and NOXA (Yip & Reed, 2008). It is thought that pro-apoptotic Bcl-2 family members form channels in the outer mitochondrial membrane through which apoptogenic proteins of the IMS are released (Korsmeyer et al, 2000).

1.2.3 Crosstalk between apoptosis signalling pathways

There is also crosstalk between the extrinsic and intrinsic apoptosis pathways. Death receptors can activate the cell-intrinsic pathway by CASP8-mediated cleavage of BID. The resulting truncated BID (tBID) interacts with the pro-apoptotic BAX and BAK, leading to their oligomerization and insertion in the outer mitochondrial membrane (Eskes et al, 2000; Wei et al, 2000). This results in release of mitochondrial CYTC and SMAC/DIABLO, as well as subsequent activation of CASP9 and 3 and amplification of apoptosis induction through the cell-extrinsic pathway. In some cell types, death-receptor engagement commits cells to apoptotic death. In other cell types, apoptosis may require amplification of the death-receptor signal by the cell-intrinsic pathway (Scaffidi et al, 1999b).



Scheme 5. Crosstalk between DR-mediated and intrinsic apoptosis. Modified after (Ashkenazi, 2002)

1.2.4 Negative regulators of apoptosis

Cells possess a complex set of mechanisms regulating their responsiveness to DR ligands and intrinsic apoptosis inducers. While most TNFR-superfamily (TNFRSF) members function as transmembrane signal transducers that respond to ligand binding, some do not signal and act as decoys that compete for the interaction of cognate ligands with their signalling receptors (Ashkenazi, 2002). Table 1 lists some examples of decoy receptors (DcRs).

Table 1. Death ligand DcRs

Ligand	DcR	Description	Reference
TRAIL	Osteoprotegerin (OPG)	Soluble decoy receptor that binds to TRAIL and protects from TRAIL-induced apoptosis	(Emery et al, 1998)
	DcR1	DcR1 lacks a cytoplasmic DD and is attached to the plasma membrane through a glycophospatidylinositol anchor	(Sheridan et al, 1997)
	DcR2	DcR2 is a transmembrane protein with a truncated DD in its cytoplasmic tail that is unable to signal apoptosis	(Marsters et al, 1997)
FasL	DcR3	DcR3 encodes a soluble protein that contains signal sequence but no transmembrane domain	(Pitti et al, 1998)

Furthermore, cells must protect themselves from inappropriate activation of apoptosis to ensure survival. To achieve this, they express a whole array of anti-apoptotic factors that block the initiation of the cytotoxic response triggered by the extrinsic or intrinsic pathway mainly at two critical levels:

- Apoptosis inhibition at the caspase level: IAPs are a broadly conserved family of anti-apoptotic proteins that block cell death, in part, by directly inhibiting the activation pathways of CASP3, 7 and 9. Over-expression of IAPs prevents apoptosis induced by a wide variety of stimuli, including TNFα, FasL, staurosporine, etoposide and growth factor withdrawal (Deveraux et al, 1998). Eight mammalian IAPs are known at present: X chromosome-linked IAP (XIAP), cellular IAP1 and IAP2 (cIAP1/2), neuronal apoptosis inhibitory protein (NAIP), SURVIVIN, BRUCE, LIVIN and testis-specific IAP (Ts-IAP) (Mannhold et al, 2010; Schimmer, 2004). The criteria for membership in this IAP family are the presence of a BIR domain and the ability to

inhibit apoptosis. BIR domains are known to be critical for the inhibitory activity of IAP proteins by providing an interaction site for caspases. Cellular FLICE-like inhibitory proteins (cFLIPs) represent another group of highly conserved anti-apoptotic proteins that act by impairing caspase activation. FLIPs function as dominant-negative inhibitors of CASP8 by using a DD to block the recruitment of proCASP8 to the DISC. As a result, CASP8 subsequent processing and activation is blocked and the pro-apoptotic signal is impaired (Budd et al, 2006).

- Apoptosis inhibition at the mitochondrial level: As mentioned previously (see 1.2.2) the mitochondria-associated BCL2 family of proteins includes anti-apoptotic members such as BCL2, BCL-X_L and BCL-w. Currently, the mechanisms by which members of the BCL2 family regulate apoptosis have not been completely understood. However, a physical interaction between pro- and anti-apoptotic BCL2 family members facilitates to circumvent the cell death process. Competition between homodimerization and heterodimerization of pro- and anti-apoptotic BCL2 family members may play a pivotal role in their capacity to induce or prevent cell death by impairing the swelling of mitochondrial membrane, maintaining the mitochondrial metabolism, limiting the effect of reactive oxygen species and regulating the MTP pore. Ultimately, all these mechanisms hinder the release of pro-apoptotic factors from the mitochondria which responsible for initiating the apoptotic cascade (Bortner & Cidlowski, 2002).

1.2.5 miRNA-mediated regulation of apoptosis

Several lines of evidence point to miRNAs as important apoptosis regulatory factors. To date, no less than 30 individual miRNAs have been experimentally demonstrated to regulate apoptosis. This number is expected to increase quickly since *in-silico* analysis predicts ~93% of all known vertebrate miRNAs to have at least one target gene related to cell death and survival (Yang et al, 2009). Depending on the nature of the targeted genes, miRNAs can be catalogued as pro- or anti-apoptotic. However, experimental evidence has shown that the effect of a given miRNA on cell death may be strongly influenced by the cellular context (Yang et al, 2009). So far the role of miRNAs in the regulation of apoptosis has been best characterized in the context of DR-independent signaling. One prominent example of an anti-apoptotic miRNA is miRNA-21, which is the most consistently up-

regulated miRNA across many cancer types (Wang & Lee, 2009). MiRNA-21 knockdown resulted in an impaired cell viability of cultured glioblastoma cells and this correlated with increased CASP3 and 7 activity and stronger TUNEL staining (Chan et al, 2005; Corsten et al, 2007). MiRNAs can also have strong pro-apoptotic features like for instance miRNA-29b which targets the anti-apoptotic BCL2-family member gene myeloid cell leukemia sequence 1 (MCL1) (Mott et al, 2007). Table 2 presents additional examples of miRNAs with known pro- or anti-apoptotic properties.

Table 2. Examples of pro- and anti-apoptotic miRNAs

Name	Effect	Target gene(s)	Reference
miRNA-34a	pro-apoptotic	E2F3	(Welch et al, 2007)
miRNA-15a and 16-1		BCL2	(Cimmino et al, 2005)
miRNA-1		HSP60/70	(Xu et al, 2007)
let-7		RAS/NF2	(Johnson et al, 2005; Meng et al, 2007)
miR-27a	anti-apoptotic	ZBTB10/RINZF	(Scott et al, 2006)
miRNA-14		DRICE	(Xu et al, 2003)
miRNA-155		TP53INp1	(Gironella et al, 2007)
miRNA-221/222		ER-α	(Zhao et al, 2008)

1.3 Pathogen recognition during innate immunity

In the course of evolution the mammalian immune system developed into a complex array of cellular and molecular components and processes that, most times, effectively shelter the organism from diseases caused by invading pathogens such as bacteria, viruses, fungi or parasites. It also plays an important role during the control of transformed "malignant self cells" that may become harmful or even lethal for the organism (Wolska et al, 2009). Protection is provided by layered defense mechanisms of increasing specificity {Murphy, 2008 #2616}. The first shield consists of unspecific physical barriers such as skin, epithelial surfaces of respiratory, urinary and gastrointestinal tracts, mucus and saliva. Pathogens breaching these are first confronted with an immediate non-specific "pre-programmed" defense response provided by cells of the innate immune system. If infection persists, the adaptive immune system is activated and provides a third layer of pathogen-specific long-

lasting protection. Despite profound differences between innate and adaptive immunity, both processes are tightly intertwined and, adequate immune function relies on their synergistic anti-pathogenic action (Dorhoi & Kaufmann, 2009; Murphy et al, 2008b).

The innate immune system is characterized by its germline-encoded ability to discriminate between self and non self molecules. Innate immune cells such as dendritic cells (DCs), macrophages and neutrophils, express various pattern-recognition receptors (PRRs) which recognize signature molecules of pathogens. These signature molecules are known as pathogen-associated molecular patterns (PAMPs), although they are also present on non-pathogenic microorganisms (Akira, 2009; Janeway & Medzhitov, 2002). PAMPs are well suited to innate immune recognition for three main reasons. First, they are invariant among microorganisms of a given class. Second, they are products of pathways that are unique to microorganisms. Third, they have essential roles in microbial physiology, limiting the ability of the microorganisms to evade innate immune recognition through adaptive evolution of these molecules (Medzhitov, 2007). Hitherto, several classes of PRRs such as Toll-like receptors (TLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and Nucleotide-binding oligomerization domain (NOD)-like receptor (NLRs) have been identified. PRRs recognize different PAMPs in various cell compartments (Table 1.).

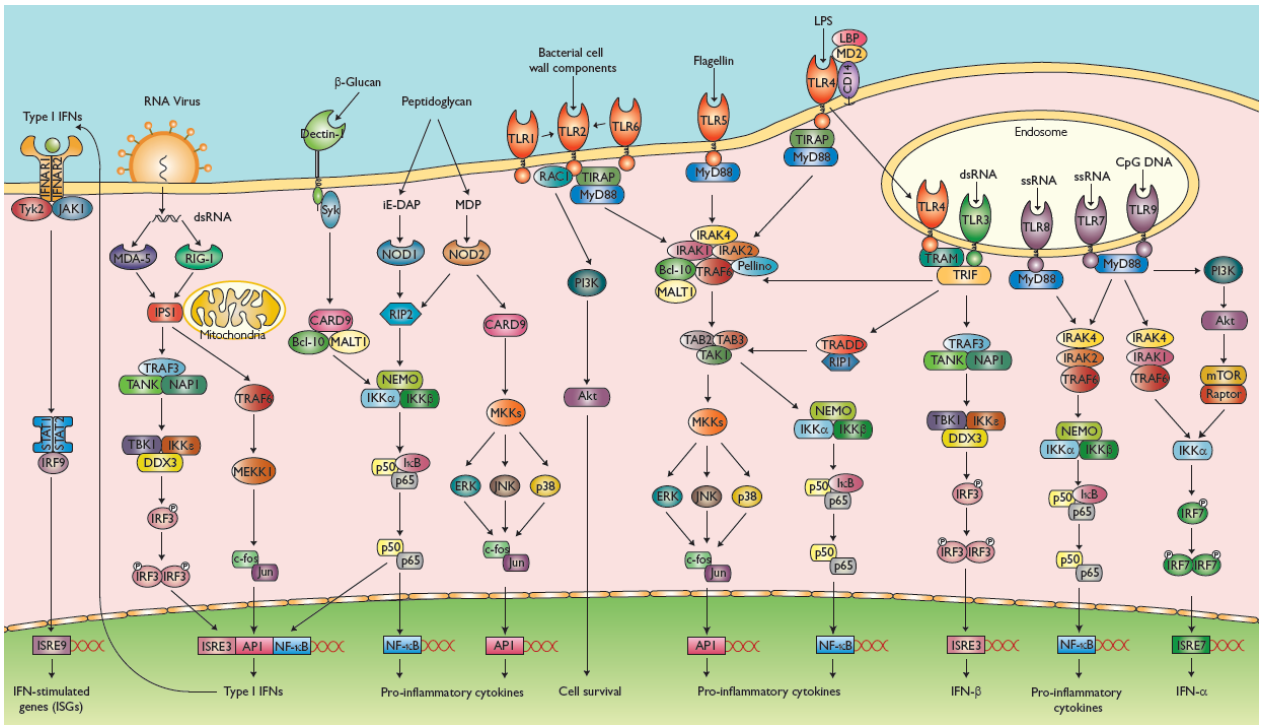
Table 3. Localization and specificity of selected human PRRs 1

Family	Name	Location	PAMPs recognized
TLRs	TLR1/2	Plasma membrane (cell surface)	Triacyl lipopeptides (bacteria and mycobacteria)
	TLR2	Plasma membrane (cell surface)	Peptidoglycan (gram-positive bacteria), LAM (mycobacteria), Hemagglutinin (measles virus), phospholipomannan (*Candida*), Glycosylphosphophatidyl inositol mucin (*Trypanosoma*)
	TLR3	Endosome	ssRNA virus (WNV), dsRNA virus (reovirus), RSV
	TLR4	Plasma membrane (cell surface)	LPS (gram-negative bacteria), Mannan (*Candida*), Glycoinositolphospholipids (*Trypanosoma*), envelope proteins (RSV)
	TLR5	Plasma membrane (cell surface)	Flagellin (flagellated bacteria)
	TLR6/2	Plasma membrane (cell surface)	Diacyl lipopeptides (mycoplasma), LTA (streptococus), Zymosan (*Saccharomyces*)
	TLR7	Endosome	ssRNA viruses (VSV, influenza virus)
	TLR8	Endosome	ssRNA from RNA virus
	TLR9	Endosome	dsDNA viruses (HSV), CpG motifs from bacteria and viruses, Hemoyoin (*Plasmodium*)
RLRs	RIG-I	Cytoplasm	Cytoplasmic 5´-triphosphate dsRNA (flavivirus, HCV)
	MDA5	Cytoplasm	Cytoplasmic dsRNA (picornavirus, ECMV)
NLRs	NOD1	Cytoplasm	g-D-glutamyl-meso-diaminopimelic acid (gram-negative and gram-positive bacteria)
	NOD2	Cytoplasm	muramyl dipeptide (gram-negative and gram-positive bacteria)
	IPAF	Cytoplasm	Flagellin (flagellated bacteria)
	NALP3	Cytoplasm	Bacterial RNA (*L. monocytogenes; S. aureus)*

[1]Based on (Chen et al, 2009; Kawai & Akira, 2009; Kumar et al, 2009; Nakhaei et al, 2009) LAM, Lipoarabinomannan; WNV, West Nile Virus; RSV, Respiratory syncitial virus; LPS, Lipopolysaccharide; LTA, Lipoteichoic acid; VSV, vesicular stomatitis virus; HSV, Herpes simplex virus and CpG, Cytidine-phosphate-guanosine.

PRR activation triggers the release of inflammatory cytokines, chemokines and type I interferons (IFNs). In the case of TLR signaling, ligand recognition leads to the recruitment of adaptor molecules such as myeloid differentiation primary response gene 88 (MyD88), Toll-interleukin 1 receptor domain-containing adaptor (TIRAP), Toll-interleukin 1 receptor domain-containing adapter protein inducing IFN-beta (TRIF) and translocation associated membrane protein 1 (TIRAP). All these adaptors share a common structural feature denominated Toll/Interleukin-1 receptor (TIR) domain and their engagement leads to activation of specific signaling pathways and transcription factors like nuclear factor 'kappa-

light-chain-enhancer' of activated B-cells (NF-κB) and members of the interferon regulatory factor (IRF) family. Furthermore, recruitment of TIR adaptor molecules also results in the activation of mitogen-activated protein kinases (MAPKs) such as p38, c-Jun N-terminal kinases (JNKs) and extracellular-signal regulated kinases (ERKs) leading to the activation of the transcription regulator activator protein 1 (AP1). These transcription factors are responsible for inducing the expression of pro-inflammatory effector molecules (Beutler, 2009b; Kumar et al, 2009; Rasmussen et al, 2009) (Scheme 6). RLRs rely on different signaling mechanisms to activate the inflammatory response. Upon recognition of dsRNA, RIG1 or melanoma differentiation associated protein-5 (MDA5) are recruited by the adaptor IFN-β promoter stimulator 1 (IPS1) to the outer membrane of the mitochondria leading to the activation of several transcription factors including IRF3, IRF7 and NF-κB (Kawai et al, 2005). IRF3 and IRF7 control the expression of type I IFNs, while NF-κB regulates the production of inflammatory cytokines (Scheme 6).



Scheme 6. Molecular signaling pathways triggered by PAMPS through PRRs (source: www.invivogen.com)

Finally, PAMP sensing through NLRs like nucleotide-binding oligomerization domain containing 1 or 2 (NOD1/2) involves the recruitment of signaling molecules including receptor-interacting protein 2 (RIP2) or caspase recruitment domain family member

9 (CARD9). These are responsible for the transmission of the signal that leads to the final activation of NF-κB and AP1 (Scheme 6).

PRR mediated secretion of inflammatory cytokines and chemokines is of central importance since it modulates and controls not only the innate immunity but also the adaptive immune response against invading pathogens. Cytokines like interleukin 1 beta (IL1β), interleukin 6 (IL6) or tumor necrosis factor alpha (TNFα) contribute in an autocrine and paracrine manner to activate the surrounding vascular epithelium at the site of infection, to prime lymphocytes and to recruit neutrophils, basophils and T cells. Chemokines are chemoattractant molecules that function by recruiting leukocytes, monocytes, neutrophils and other effector cells to the site of infection. Some prominent examples include interleukin 8 (IL8) and chemokine (C-C motif) ligand 5 (CCL5 also known as RANTES). In case of viral infections also type I IFNs *i.e.* IFN-alpha and beta (IFNα/β) are produced and released by infected cells. These contribute mainly through three different mechanisms to the inhibition of viral replication. First, they induce genes that impair the expression of viral transcripts and degrade them. Second, IFNs increase the ability of infected cells to present viral peptide fragments via major histocompatibility class I (MHCI) pathway. Finally, IFNs are very potent activators of natural (NK) killer cells that can kill virus infected cells selectively. As mentioned previously, the effector molecules produced during innate immune responses are not only important because of their direct anti-pathogenic effects, but also because they contribute to the establishment and modulation of antigen-specific adaptive immunity. One example of this crosstalk between innate and adaptive immunity deals with the ability of PRR-induced cytokines to enhance antigen presentation by professional antigen presenting cells (APCs). The adaptive immune response is dependent on co-stimulatory surface molecules, including CD40, CD80 and CD86, which are expressed by professional antigen-presenting cells, including macrophages and DCs cells. These molecules help to generate a mitogenic response within T cells that are exposed to antigen in the context of MHCI and II proteins. It is now clear that the key event in up-regulation of co-stimulatory molecules is the activation of a type I IFN response mediated by PPRs (Beutler et al, 2006; Hoebe et al, 2003).

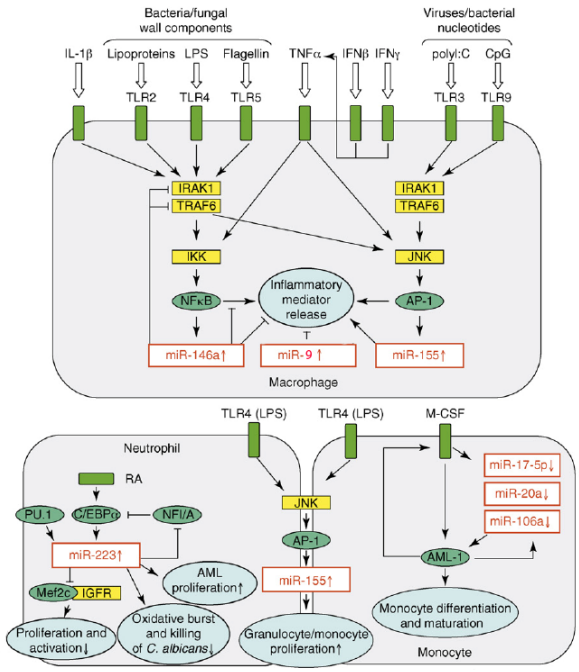
Given the pivotal importance of PRR-mediated signaling for both innate and adaptive immunity, the molecular mechanism responsible for the pro-inflammatory signals must be strictly regulated. In fact, uncontrolled regulation of TLR-mediated signaling may lead to

excessive or persistent inflammation and severe immune pathology to the host (Manicassamy & Pulendran, 2009). Several diseases including septic shock, autoimmunity, atherosclerosis, metabolic syndrome and gastric cancer have been linked to chronic or acute inflammatory responses (Beutler, 2009a; Karin et al, 2006). To date, several negative regulators of TLR-mediated inflammatory response have been identified. Notably, most of these molecules function by providing negative feedback to the signaling cascades, since they are induced upon TLR activation and impair the response at multiple levels. Some examples of negative regulators include phosphatidylinositol-3 kinase (PI3K), suppressor of cytokine signaling 1 (SOCS1) and proteins such as IRAK-M und A20 (Boone et al, 2004; Dalpke et al, 2008; Fukao et al, 2002; Kobayashi et al, 2002). The relevance of these mechanisms is underlined by the fact that mutations affecting the expression of these genes are related to human health disorders like early-onset persistent asthma, coronary artery disease in type 2 diabetes and adult asthma (Balaci et al, 2007; Boonyasrisawat et al, 2007; Harada et al, 2007).

1.3.1 miRNA-mediated regulation of innate immune responses

Inflammatory responses to invading pathogens involve the induction of numerous genes, a process that must be tightly regulated to achieve pathogen clearance and at the same time avoid consequences of dysregulated gene expression such as uncontrolled inflammation and cancer (Sonkoly et al, 2008). The involvement of miRNAs in the fine tuning of innate immunity has been a field of intensive research. This has led to the identification of miRNAs as important regulators of monocyte differentiation and maturation, granulocyte proliferation and activation, pathogen sensing, inflammatory responses and antiviral immunity (Fontana et al, 2007; Jing et al, 2005; Johnnidis et al, 2008; Pedersen et al, 2007; Taganov et al, 2006). In macrophages it has been shown that activation of TIRs and TNFα receptor results in rapid expression of host miRNAs such as miRNA-9, 146a and 155 (Bazzoni et al, 2009; O'Connell et al, 2007; Perry et al, 2008; Taganov et al, 2006). As revealed by target gene analysis, these impair the expression levels of proteins involved in the pro-inflammatory signaling pathway including TNF receptor-associated factor 6 (TRAF6) (miRNA-146a), interleukin-1 receptor-associated kinase 1 (IRAK1) (miRNA-146a), NF-κB1 (miRNA-9), NF-κB inhibitor epsilon (IKKε) (miRNA-155) and mitogen-activated protein kinase kinase kinase 7 interacting protein 2 (TAB2) (miRNA-155) (Bazzoni et al, 2009; Ceppi et al, 2009; Taganov et al, 2006; Xiao et al, 2009). MiRNA-mediated down-regulation of these genes allows cells to control

their activation status by dampening the signaling pathways that govern the expression of pro-inflammatory cytokines. Innate immunity-associated miRNAs perform also functions other than controlling the acute response to invading pathogens (Scheme 7).



Scheme 7. Role of microRNAs in the innate immune response. Modified after (Lindsay, 2008)

In humans, analysis of monocyte differentiation showed that stimulation of cord blood $CD34^+$ with macrophage-colony stimulating factor (M-CSF) resulted in reduced expression of miRNA-17-5p, 20a and 106a. Further experiments revealed that decreased expression of these miRNAs allowed higher levels of their target protein acute myeloid leukemia-1 (AML1) to occur. AML1 is a transcription factor responsible for induction of the M-CSF receptor, IL13 and granulocyte macrophage-colony stimulating factor (GM-CSF) (Fontana et al, 2007; Tsitsiou & Lindsay, 2009). Interestingly, miRNA-155 may also drive the expansion of granulocyte and macrophage populations. Indeed long-term expression of miRNA-155 in hematopoietic stem cells and engraftment into lethally irradiated mice resulted in a phenotype similar to acute myeloid leukemia (AML) (O'Connell et al, 2008) (Scheme 7). An additional well-studied example of the importance of miRNA-mediated regulation of the innate immune system is provided by miRNA-223. Expression profiling has shown that miRNA-223 is transcribed in myeloid cells in the bone marrow and that its increased expression is relevant for the differentiation of precursor cells into granulocytes (Chen et al, 2004; Fazi et al, 2005;

Johnnidis et al, 2008). It was suggested that miRNA-223 exerts its role through the down-regulation of genes such as myeloid ELF1-like factor (MEF2c), insulin-like growth factor receptor (IGFR) and nuclear factor I-A (NFI-A) (Fazi et al, 2005; Johnnidis et al, 2008) (Scheme 7). Providing further evidence for the multiple roles of miRNAs, neutrophils from miRNA-223 knockout mice showed enhanced oxidative burst and killing of *Candida albicans*. Moreover, these mice spontaneously developed inflammatory lung pathology and exhibited exaggerated tissue destruction after endotoxin challenge. This suggests that miRNA-223 can also act as a negative regulator of the inflammatory response (Johnnidis et al, 2008).

1.4 Tuberculosis

Mycobacterium tuberculosis (M. tuberculosis), the causative agent of human tuberculosis (TB), kills nearly two million people annually and has been a major health threat for centuries (Parida & Kaufmann, 2010; World Health Organization., 2009). TB is a disease of poverty with the highest incidence rates occurring in low income and lower middle-income countries. Despite its importance as a global health threat, the most recent TB drug was developed decades ago; the standard diagnostic technology used in developing countries is more than 100 years old; and the BCG vaccine, with almost no protective effect in adults, was introduced in 1921 (Aagaard et al, 2009; Murphy et al, 2008a). In the post-genomic era, innovative tools that help us win the fight against this terrifying disease are urgently needed.

1.4.1 History and biology

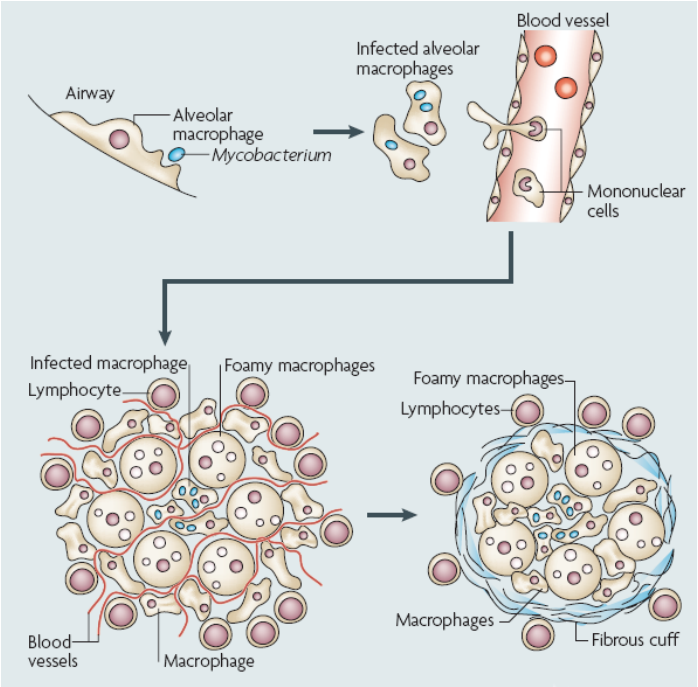
TB is a millennial disease that has affected and shaped human civilizations since their very early beginnings. As demonstrated by palaeopathologic studies, Neolithic settlements from 9250-8160 years ago, around the time of the first great transition from hunter-gatherers to a settled agriculture-based lifestyle, and predynastic Egyptians (3500-2650 B.C.) suffered the inclemency of TB (Hershkovitz et al, 2008; Zink et al, 2001). Accordingly, numerous descriptions of TB are found in ancient texts (Daniel, 2006). The first description of *M. tuberculosis* as the causative agent of human TB was by Robert Koch on March 24th, 1882. His groundbreaking discovery was honored with the Nobel Prize in physiology or medicine in 1905 (Kaufmann & Schaible, 2005). Thereafter, further advancements in the fight against

TB were achieved. By 1921, Albert Calmette and Camille Guérin developed a viable attenuated vaccine. In 1943 Selman A. Waksman identified Streptomycin as the first antituberculous drug, opening the window for chemotherapeutic treatment of the disease (Kaufmann, 2005; Waksman, 1954). Despite these and other scientific breakthroughs in TB research, difficulties associated with the chemotherapeutic treatment, a vaccine that protects children but not adults, the appearance of human immunodeficiency virus (HIV) and the advent of drug-resistant strains kept TB as an ongoing threat. In 1993, the WHO declared TB as global emergency facing and accepting the fact that there is more TB in the world today that at any other time in history.

M. tuberculosis is a slowly growing obligate aerobe bacterium. One central hallmark of this human pathogen is its cell envelope which contains an additional layer beyond the peptidoglycan that is exceptionally hydrophobic and rich in unusual lipids, glycolipids and polysaccharides (Brennan, 2003). In 1998 the sequencing of the complete *M. tuberculosis* genome revealed a very high guanine and cytosine content and ~ 4000 genes, from which a significant portion is devoted to the production of enzymes involved in lipogenesis and lipolysis (Cole et al, 1998). Importantly, several lines of evidence have demonstrated that this enhanced lipid metabolism is of crucial importance for the manipulation of the host cells during infection (Guenin-Mace et al, 2009).

In TB, the lung is typically the port of entry and site of active disease although virtually all other organs can be afflicted (Kaufmann, 2006). Infection occurs via inhalation of droplets containing bacilli that are released by individuals with active pulmonary TB. In the lung, inhaled *M. tuberculosis* is ingested by resident alveolar macrophages and DCs. Inside these cells bacilli block the phagosome maturation by interrupting acidification and lysosome fusion, which creates a protected niche in the cell for bacterial replication (Armstrong & Hart, 1971). Infected phagocytes serve as Trojan horses that transport mycobacteria to the draining lymph nodes, were presumably secreted proteins such as early secreted antigen for T cells (ESAT6) and antigen 85 (Ag85) are presented to $CD4^+$ T cells (Kaufmann & McMichael, 2005). Infected macrophages that remain in the lung induce a local pro-inflammatory response that leads to the recruitment of mononuclear cells from neighboring blood vessels. They also interact with activated antigen-specific T cells giving rise to the formation of a productive granulomatous lesion which is composed of infected

macrophages surrounded by foamy macrophages and other mononuclear phagocytes mantled by a layer of lymphocytes in association with a fibrous cuff of collagen and other extracellular matrix components that delineate the periphery of the structure (Russell, 2007) (Scheme 8).



Scheme 8. Granuloma formation after airborne M. tuberculosis infection. Modified after (Russell, 2007)

The granuloma deprives the arrested mycobacteria of oxygen and nutrients forcing the microbes to survive in a state of dormancy. Granuloma formation takes place in over 90% of infection cases and is a hallmark of the so called "containment" or "latent" phase in which there are no disease symptoms and the host is not contagious for others (Kaufmann & McMichael, 2005). Latent infection can be maintained for the lifetime of the host. Reactivation occurs in 5 to 10% of infected persons, and can be triggered by immunosuppression due to age, corticosteroids, malnutrition or other factors (Flynn, 2004). Consequently, co-infection with HIV increases the risk of developing TB several 100-fold (Kaufmann & McMichael, 2005). When individuals with active pulmonary TB cough, sneeze, speak, or spit, they expel infectious aerosol droplets. A single sneeze can release up to 40 000 droplets (Cole & Cook, 1998). Importantly, each single droplet represents significant transmission risk, since TB infectious dose is very low and inhaling less than ten bacteria may cause an infection (Behr et al, 1999; Nicas et al, 2005).

1.4.2 Innate immune response of the host to TB

As mentioned previously (see 1.5.1), phagocytic cells play a key role in the initiation and direction of immune responses against mycobacteria. A number of receptors are crucial for the recognition of *M. tuberculosis* by phagocytes. These include the complement receptors, mannose receptor (MR), DC-specific intercellular adhesion molecule-3 (ICAM3)-grabbing non-integrin (DC-SIGN), surfactant protein A (SP-A) receptor, the class A scavenger receptor and mannose-binding lectin (MBL) (Berrington & Hawn, 2007). Whereas human macrophages use MR and complement receptor 3 (CR3) for binding to *M. tuberculosis*, DCs primarily use DC-SIGN (Kang et al, 2005; Schlesinger, 1993; Tailleux et al, 2003). Also TLRs have been associated with the recognition of *M. tuberculosis*. Several *in-vitro* studies have provided evidence for TLR1, 2, 4, 6 and 9 as important sensors of tubercle bacilli (Heldwein & Fenton, 2002; Means et al, 1999; Underhill et al, 1999). Mycobacterial TLR ligands include lipoproteins (TLR2), mycolylarabinogalactan-peptidoglycan complex (TLR2), lipids (TLR2), LAM (TLR2), triacylated lipopetides (TLR1), diacylated lipopetides (TLR6), 19-kDa lipoprotein (TLR1) and a non-canonical TLR4 ligand (Berrington & Hawn, 2007). The *in-vivo* significance of individual TLRs has been more difficult to demonstrate. In the murine TB model, several studies have shown that mice deficient in MyD88 are highly vulnerable to intranasal TB infection and die due to unrestrained bacterial growth (Fremond et al, 2004; Scanga et al, 2004; Sugawara et al, 2003). On the other hand, the data concerning mycobacterial challenge of TLR2, 4, 9 single, TLR2/9 double and TLR2/4/9 triple knockout mice has been less conclusive, ranging from normal to increased susceptibility (Korbel et al, 2008). In humans, several genetic studies indicate that TLR pathway variants may influence susceptibility to disease. These include polymorphisms in the TIR domain of TLR2 and TIR domain containing adapter protein (TIRAP) (Khor et al, 2007; Ogus et al, 2004).

M. tuberculosis recognition by macrophages results in the induction of a large number of cytokines, some of which have been demonstrated to be essential for the proper control of TB. TNFα is strongly produced after exposure of monocytes and macrophages to mycobacterial products. In synergy with IFNγ, TNFα activates macrophages to produce nitric oxide synthase 2 (NOS2), allowing infected macrophages to eliminate intracellular bacteria (Flesch et al, 1994). Indeed, TNFα and TNFR knockout mice are characterized by higher susceptibility to *M. tuberculosis* and impaired granuloma formation (Bean et al, 1999; Flynn

et al, 1995a). Furthermore, human patients receiving inhibitors of TNFα are more susceptible to develop TB (Keane et al, 2001). Also IL1β, a central pro-inflammatory molecule, has been associated with an effective immune reaction to invading *M. tuberculosis.* Mice that lack both IL1β and α develop larger granulomas and show impaired bacterial clearance when compared to wildtype animals (Yamada et al, 2000). Consistently, IL1R knockout mice are characterized by impaired survival and succumb to exaggerated mycobacterial growth (Juffermans et al, 2000). Immunologic control of *M. tuberculosis* infection relies on a Type 1 T-cell response. IL12 is strongly induced following phagocytosis of tubercle bacilli by macrophages and DCs and skews the response to a T-helper (TH) 1 IFNγ producing phenotype (Ladel et al, 1997). IL12 is so important for the immune response against *M. tuberculosis*, that its exogenous administration to mice can improve survival after bacterial challenge (Flynn et al, 1995b). Accordingly, $IL12^{-/-}$ mice and humans with mutations of the IL12 system are more susceptible to mycobacterial infection (Cooper et al, 1997; Ottenhoff et al, 1998). As mentioned previously, IFNγ is a central cytokine for macrophage activation and the control of *M. tuberculosis* infection. The strongest line of evidence supporting this is provided by IFNγ knockout mice that are the most susceptible mouse strain to virulent *M. tuberculosis* (Cooper et al, 1993; Flynn et al, 1993). Moreover, individuals defective in genes for IFNγ or its receptor succumb easier to mycobacterial infection (Ottenhoff et al, 1998).

Not only macrophages and DCs, but also other innate immune cells play an important role during the defense response of the host to *M. tuberculosis.* Natural killer (NK) cells are granular lymphocytes of the innate system (Moretta et al, 2008). Human NK cells express granulysin a peptide that has been shown to kill *M. tuberculosis* (Stenger et al, 1999). Human NK cells not only lyse infected cells but also actively restrict mycobacterial propagation in an apoptosis-dependent but Fas/FasL-independent manner (Brill et al, 2001). *In-vivo* infections have shown that NK cells accumulate two weeks after challenge with *M. tuberculosis* in the lungs of mice and secrete IFNγ, and that NK cell depletion prior and during infection does not affect control of mycobacterial growth (Junqueira-Kipnis et al, 2003). This result implies that even though NK cells are able to respond to pathogenic mycobacterial challenge, their functions must overlap with other immune cells. Neutrophils constitute the first line of defense of the innate immune system, phagocytosing and killing pathogens through oxygen-dependent and/or independent mechanisms, and neutrophil extracellular trap (NET) formation (Brinkmann et al, 2004; Segal, 2005; Urban et al, 2009; Urban et al, 2006). For a

long time the role of neutrophils during *M. tuberculosis* infection was neglected. During the last years evidence has accumulated suggesting that neutrophils have a function during the acute phase of infection and early granuloma formation (Barrios-Payan et al, 2006; Pedrosa et al, 2000; Seiler et al, 2003). More recently, two strains of the *M. tuberculosis* complex of varying virulence, *M. tuberculosis* H37Rv and *M. canettii*, were shown to induce subcellular changes leading to NET formation that captured mycobacteria but were unable to kill them (Ramos-Kichik et al, 2009). Despite all these information, the role of neutrophils during mycobacterial infection remains controversial. An important open question here is whether neutrophils control the spread of the pathogen by killing *M. tuberculosis* or if they rather contribute to development of the pathology.

Despite all this accumulating knowledge about cellular mechanisms and molecules imperative for innate immunity against *M. tuberculosis,* a person dying every 20 seconds of TB still reminds us how limited our understanding of this devastating disease is (World Health Organization., 2009). Better innovative treatments will be only possible if we get to know more about the basic biology of this devastating disease (Kaufmann, 2006).

1.5 Aims of this study

Since their discovery in the early 90´s miRNAs have revolutionized our understanding of gene activity regulation (Bartel, 2004; Lee et al, 1993; Wightman et al, 1993). Their significant role in the control of gene expression has been described in many different biological processes including development of the immune system and orchestration of anti-pathogenic responses (Taganov et al, 2007). Despite the crucial relevance of miRNAs in the interplay between host and pathogen currently nothing is known about the role of miRNAs during the immune response to *M. tuberculosis.* This doctoral thesis aimed at gaining an insight into the immunoregulatory function of miRNAs during mycobacterial infection. To this end, the following objectives were pursued:

- Generation of a miRNA expression profile of THP1 cells infected with mycobacterial strains of varying virulence. Identification and validation of differentially regulated miRNAs.

- Identification of miRNAs with pro- or anti-apoptotic properties using an experimental *ex-vivo* model of DR-mediated apoptosis.

- Characterization of the exact molecular mechanisms responsible for pro- or anti-apoptotic miRNA-mediated phenotypes. Identification of miRNA target genes using a combined approach (*i.e*. microarray expression analysis, pSILAC and *ex-vivo* targeting assays) to detect expression changes at both the transcriptional and translational levels.

- Dissection of the mechanisms controlling the expression of pro- or anti-apoptotic miRNAs. Assessment of their involvement in the regulation of innate immune responses.

- Construction of lentiviral miRNA over-expression constructs for the generation of stably transduced macrophages. Characterization of the effects of miRNA over-expression on the innate immune response to *M. tuberculosis*

Taken together, this study shall contribute to a better understanding of the innate immune response mounted by macrophages infected with mycobacterial pathogens and the regulation of this process by miRNAs.

2. Material & Methods

2.1 Material

2.1.1 Biochemicals and molecular biological reagents

Name	Supplier
1 kb plus ladder	Invitrogen (Carlsbad, CA, USA)
$^{13}C_6$ $^{15}N_4$ L-Arginine-2HCl	Perbio Science (Erembodegem, Belgium)
$^{13}C_6$ L-Arginine-HCl	Perbio Science (Erembodegem, Belgium)
$^{13}C_6$, $^{15}N_2$ L-Lysine-2HCl	Perbio Science (Erembodegem, Belgium)
7-Amino-actinomycin (7-AAD)	Immunochemistry Technologies (Bloomington, MN, USA)
AccuPrime *Taq* Polymerase High Fidelity	Invitrogen (Carlsbad, CA, USA)
Acrylamide/Bis-Acrylamide	Bio-Rad Laboratories (Hercules, CA, USA)
Agarose LE	Biozym (Oldendorf, Germany)
Anti-Fas (Clone CH11)	Upstate (Temecula, CA, USA)
Anti-miRNAs	Ambion (Austin, TX, USA)
Cyclohexamide	Sigma Aldrich (St. Louis, MO, USA)
FastSYBR® Green master mix	Applied Biosystems (Carlsbad, CA, USA)
FLICA® CASP3	Immunochemistry Technologies (Bloomington, MN, USA)
FLICA® CASP8	Immunochemistry Technologies (Bloomington, MN, USA)
Glycogen	Ambion (Austin, TX, USA)
Lipofectamine 2000®	Invitrogen (Carlsbad, CA, USA)
L-Lysine-4,4,5,5-d_4 hydrochloride	Perbio Science (Erembodegem, Belgium)
Negative ctrl. miRNA and anti-miRNA #1	Ambion (Austin, TX, USA)
Osteoprotegerin-Fc	Biomol (Hamburg, Germany)
PageRuler® Plus Prestained Protein Ladder	Fermentas (Burlington, Canada)
PMA (phorbol-12-myristate-13-acetate)	Cell Signaling (Danvers, MA, USA)
Pre-miRNAs	Ambion (Austin, TX, USA)
Propidium iodide	Immunochemistry Technologies (Bloomington, MN, USA)
Random hexamers	Fermentas (Burlington, Canada)
SILAC MEM PRO *LYS (D-MEM) KIT	Invitrogen (Carlsbad, CA, USA)
Staurosporine	Sigma Aldrich (St. Louis, MO, USA)
Super*Killer*® TRAIL	Alexis Biochemicals (Farmingdale, NY, USA)
SuperSignal West Pico Chemiluminescent Substrate	Pierce (Rockford, IL, USA)
TNFα	Active Bioscience (Hamburg, Germany)
TOPO Cloning kit	Invitrogen (Carlsbad, CA, USA)
Trizol	Invitrogen (Carlsbad, CA, USA)
Z-VAD-FMK	BD-Biosciences (San Jose, CA, USA)

2.1.2 Antibodies

Table 5. Antibodies used for Western blot analysis of protein expression

Specificity	Host species	Clone	Dilution	Supplier
Anti-mouse IgG HRP-linked	goat	polyclonal	1:3000	Cell Signaling (Danvers, MA, USA)
Anti-rabbit IgG HRP-linked	goat	polyclonal	1:3000	Cell Signaling (Danvers, MA, USA)
cFLIP	Rabbit	polyclonal	1:1000	Cell Signaling (Danvers, MA, USA)
FAIM-s	Rabbit	polyclonal	1:5000	AntibodyBcn (Barcelona, Spain)
FASN	Rabbit	polyclonal	1:1000	Cell Signaling (Danvers, MA, USA)
GAPDH	Rabbit	14C10	1:1000	Cell Signaling (Danvers, MA, USA)
GSTP1	Mouse	3F2	1:1000	Cell Signaling (Danvers, MA, USA)
PARP	Rabbit	polyclonal	1:1000	Cell Signaling (Danvers, MA, USA)

2.1.3 Primers for 3´-UTR cloning

Following primers were used for generation of luciferase reporter constructs for detecting miRNA-mediated silencing. The amplified fragments encode the full length 3´-UTR of the candidate target genes. For cloning purposes the XhoI and NotI restriction sequences were added to the forward (fw) and reverse (re) primer respectively.

Table 6. Primers used for generation of 3´-UTR luciferase reporter constructs

Primer Name	Primer sequence 5´→ 3´
FAIM-3´-UTR _fw	**ctcgag**[1] TGAATTTTCATCTTAAGAAGTAAAGATCAG
FAIM-3´-UTR _re	**gcggccgc**[2] AAAAGTATTTATTATAGTAAAGGTTACTGTTGT
FASN-3´-UTR _fw	**ctcgag** CTGCCACCGGAGGTCACT
FASN-3´-UTR _re	**gcggccgc** GGGAGGCTGAGAGCAGCA
FASN-from_cds _fw	**ctcgag** GCCCAGGTGGAGGACG
FASN-from_cds _re	**gcggccgc** ATCTCTCAAGACCACGGCC
GSTP1-3´-UTR _fw	**ctcgag** AGTGAGGGTTGGGGGGA
GSTP1-3´-UTR _re	**gcggccgc** AGCTCTCTTAGAAATTTTATTGGTCct
OPG-3´-UTR _fw	**ctcgag** CTGGAAATGGCCATTGAGC
OPG-3´-UTR _re	**gcggccgc** AAGGTACATTCAATTTCTCAGAGCA

[1] XhoI restriction sequence

[2] NotI restriction sequence

2.1.4 Primers for gene expression quantification

Expression levels of osteoprotegerin (OPG), Fas-apoptosis inhibitory molecule (FAIM), fatty acid synthase (FASN), glutathione S-transferase pi 1 (GSTP1) were determined using commercially available mRNA specific Quantitect primer assays (Qiagen, Hilden, Germany). For quantification of interleukin 6 (IL6), 8 (IL8) and human acidic ribosomal protein (HuPO) the primers listed below were used. Oligonucleotides were designed to span across exons and specifically amplify mRNA transcripts. They were purchased from MWG Biotech (Ebersberg, Germany).

Table 7. Detection primers for quantification of gene expression

Primer Name	Primer sequence 5´→ 3´
HuPO_fw	GCTTCCTGGAGGGTGTCC
HuPO_re	GGACTCGTTTGTACCCGTTG
IL6_fw	CAATCTGGATTCAATGAGGAGAC
IL6_re	CTCTGGCTTGTTCCTCACTACTC
IL8_fw	GAACTGAGAGTGATTGAGAGTGGA
IL8_re	CTCTTCAAAAACTTCTCCACAACC

2.1.5 Plasmids

Following plasmids were used for the experiments presented here. All luciferase expressing plasmids were generated from Promega (Madison, WI, USA). pCR2.1-TOPO® was obtained from Invitrogen (Carlsbad, CA, USA). pGL3.ELAM.tk was a kind gift Dr. Juana de Diego (Max-Planck Institute for Infection Biology, Berlin, Germany).

Table 8. Plasmids

Name	Description
pCR2.1-TOPO®	Linearized vector with single 3´thymidine (T) overhangs for TA cloning and Topoisomerase I covalently bound to the vector
pGL3.ELAM.tk	Encodes a luciferase cloned from *Photinus pyralis* and driven by an NF-κB-responsive element from the E-selectin gene
pGL3-control	Encodes a luciferase cloned from *Photinus pyralis* and expressed under the control of SV40 early enhancer/promoter region (Yang et al, 1998)
pRL-SV40	Encodes a luciferase cloned from *Renilla reniformis* and expressed under the control of SV40 early enhancer/promoter region
psiCHECK-2	Expresses two luciferases cloned from *Renilla reniformis* (SV40 early enhancer/promoter) and *Photinus pyralis* (HSV-TK promoter)

2.1.6 Toll-like receptor (TLR) ligands

For stimulation of TLRs the human TLR1-9 agonist kit from Invivogen (San Diego, CA, USA) was purchased. Lyophilized material was resuspended using endotoxin-free water according to the manufacturer´s instructions. Dissolved reagents were aliquoted and stored at -80°C.

2.1.7 Mycobacterial strains and culture

Infection experiments of human macrophages were performed under BL3 conditions with the virulent *Mycobacterium tuberculosis* H37Rv (*M. tuberculosis* H37Rv) lab strain obtained from American Type Culture Collection (Manassas, VA, USA). Recombinant eGFP expressing *Mycobacterium bovis* BCG (*M. bovis* BCG) was kindly provided by Dr. Nathalie Winter from mycobacterial genetics unit (Institute Pasteur, Paris, France). Bacterial cultures were grown in Middlebrook 7H9 broth (see 2.1.8).

2.1.8 Eukaryotic cell lines

HeLa and THP-1 cells were obtained from the German Collection of Microorganisms & Cell Cultures (DSMZ) (Braunschweig, Germany) and the American Type Culture Collection (ATCC) (Manassas, VA, USA), respectively. HeLa and THP-1 cells were cultivated in DMEM and complete RPMI (see 2.1.8) at 37°C and 5% CO_2 in a humidified incubator. After 15 passages cells were discarded and replaced by a new cryostocks.

2.1.9 Buffers, solutions and culture media

Middlebrook 7H9 broth:	4.7 g DifcoTM Middlebrook 7H9 powder 2 ml Glycerol 900 ml ddH_2O 100 ml BBL Middlebrook 10% (v/v) ADC enrichment 0.05% (v/v) Tween 80

DMEM for HeLa cells:
DMEM
10%(v/v) heat-inactivated fetal bovine serum
2 mM L-Glutamine
100 U/ml Penicillin
100 µg/ml Streptomycin
sterile filtered

pSILAC DMEM "light":
SILAC DMEM
10%(v/v) dialyzed heat-inactivated fetal bovine serum
2 mM L-Glutamine
2 mM L-Arginine
2 mM L-Lysine
100 U/ml Penicillin
100 µg/ml Streptomycin
sterile filtered

pSILAC DMEM "med.-heavy":
SILAC DMEM
10%(v/v) dialyzed heat-inactivated fetal bovine serum
2 mM L-Glutamine
2 mM $^{13}C_6$ L-Arginine-HCl
2 mM L-Lysine-4,4,5,5-d_4 hydrochloride
100 U/ml Penicillin
100 µg/ml Streptomycin
sterile filtered

pSILAC DMEM "heavy":
SILAC DMEM
10%(v/v) dialyzed heat-inactivated fetal bovine serum
2 mM L-Glutamine
2 mM $^{13}C_6$ $^{15}N_4$ L-Arginine-2HCl
2 mM $^{13}C_6$, $^{15}N_2$ L-Lysine-2HCl
100 U/ml Penicillin
100 µg/ml Streptomycin
sterile filtered

Complete RPMI:
RPMI
10% heat-inactivated fetal bovine serum
2 mM L-Glutamine
100 U/ml Penicillin
100 µg/ml Streptomycin
55 µM 2-mercaptoethanol
100 mM Hepes
1mM sodium pyruvate
100 U/ml Penicillin
100 µg/ml Streptomycin
sterile filtered

FLICA washing buffer:
1% BSA (w/v)
in PBS

MTT solution:	5 mg/mL MTT in PBS
1× PAGE running buffer:	25 mM Tris Base 192 mM Glycine 0,1 % (w/v) SDS pH ~ 8,5
3×Laemmli buffer:	52,8 mM Tris-HCl, pH 6,8 6 % (w/v) SDS 30 % (v/v) Glycerol 15 % (v/v) β-mercaptoethanol 0,024 % (w/v) Bromphenol blue
5 % stacking gel:	125 mM Tris-HCl, pH 6,8 5 % (w/v) Acrylamide/Bis-Acrylamide (37,5:1) 0,1 % (w/v) SDS 0,25 % (v/v) TEMED 0,1 % (w/v) APS
10 % SDS resolving gel :	375 mM Tris-HCl, pH 8,8 10 % (w/v) Acrylamide/Bis-Acrylamide (37,5:1) 0,1 % (w/v) SDS 0,15 % (v/v) TEMED 0,05 % (w/v) APS
1×Western blot buffer:	25 mM Tris base 192 mM Glycine 10 % (v/v) Methanol pH ~ 8,5
Stripping buffer:	25 mM glycine 1 % (w/v) SDS pH 2,0
1x oligo annealing buffer:	10 mM Tris-HCl (pH 7.5) 100 mM NaCl 1 mM EDTA
1×TAE:	40 mM Tris base 20 mM glacial acetic acid 1 mM EDTA
1×TBS:	25 mM Tris-HCl 123 mM Sodium chloride 5 mM Potassium chloride 0,7 mM Calcium chloride-2·H_2O 0,5 mM Magnesium chloride-6·H_2O 0,6 mM Di sodium hydrogenphosphate-2·H_2O pH 7,4

1×TBS/T:	1×TBS 0,1 % (v/v) Tween-20

2.2 Methods

2.2.1 Infection of THP-1 macrophages

Infection experiments were performed in 6-well plates. In order to induce cell adherence and differentiation 10^6 THP-1 suspension cells per well were stimulated overnight in culture medium (see 2.1.7) with 50 ng/ml PMA. Following stimulation, cell culture supernatant was discarded and adherent cells were rinsed five times with fresh medium in order to remove any remaining PMA traces. Subsequently two milliliters of medium were added to the cells and these were kept in culture for another 24 hrs. After this time, cell monolayers were washed once more with culture medium and then cultured for two more days in order to allow the cells to fully differentiate into macrophage-like cells. Second or third passage bacterial cultures in the exponential growth phase (OD_{600} = 0,7) were used for infection. The inoculum was prepared by sedimenting mycobacterial cultures via centrifugation at 3000 rpm for 10 min and washing them once with 10 ml PBS in order to dilute any remaining culture broth. Resuspended bacteria were sedimented again and then resuspended in 3 ml PBS. In order to avoid clump formation, the bacterial suspension was passed ten times through a 25 gauge needle and finally filtered using 40µm cell filters from BD-Biosciences (San Jose, CA, USA). Cells were infected in complete culture medium without antibiotics with a final multiplicity of infection (MOI) of 20:1 or 3:1 with *M. bovis* BCG or *M. tuberculosis* H37Rv, respectively. After inoculation, the culture plates were centrifuged 5 min at 1500 rpm in order to sediment the mycobacteria and facilitate phagocytosis. Infected cells were cultured four hours after which the supernatant was removed and cells were washed three times with complete medium without antibiotics. After the last washing step fresh medium was added and cells were incubated for another 24 or 48 hrs. at 37°C and 5% CO_2 in a humidified incubator.

2.2.2 Transient transfection

Transient transfection of HeLa cells was performed in 24-well format using Optimem serum-free medium and Lipofectamine2000® both from Invitrogen (Carlsbad, CA, USA). The day prior to the experiment $5*10^4$ cells were seeded per well. Transfection of the cells was performed as per manufacturer´s recommendations.

2.2.3 Bacterial transformation

One Shot® TOP10 chemically competent *E. coli* from Invitrogen (Carlsbad, CA, USA) were transformed according to manufacturer's instructions. Cryostocks were generated by storing transformed cells in LB with 7.5% Glycerol at -80°C.

2.2.4 Microarray analysis

Microarray experiments were performed as dual-color hybridizations. To compensate for dye-specific effects, a dye-reversal color-swap was applied (Churchill, 2002). Quality control and quantification of total RNA amount was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and a NanoDrop 1000 spectrophotometer (Kisker, Steinfurt, Germany). RNA labeling was performed with the Quick Amp Labeling Kit (Agilent Technologies). In brief, mRNA was reverse transcribed and amplified using an oligo-dT-T7-promotor primer. The obtained resulting cRNA was labeled with either Cyanine 3-CTP or Cyanine 5-CTP. After precipitation, purification and quantification, 1.25 µg of each labeled cRNA was fragmented and subsequently hybridized to whole human genome 44k microarrays (AMADID-014850) according to the supplier's protocol (Agilent Technologies). Hybridized microarrays were washed using the SSC washing protocol (Agilent Technologies). Scanning of microarrays was performed with 5 µm resolution using a DNA microarray laser scanner (Agilent Technologies). Raw microarray image data were analyzed with the Image Analysis / Feature Extraction software G2567AA (Version A.9.5.1, Agilent). The extracted MAGE-ML files were further analyzed with the Rosetta Resolver Biosoftware, Build 7.2 (Rosetta Biosoftware, Seattle, WA, USA). Ratio profiles comprising single hybridizations were combined in an error-weighted fashion to create ratio experiments. A 1.5 fold-change expression cut-off for ratio experiments was applied together with anti-correlation of ratio profiles.

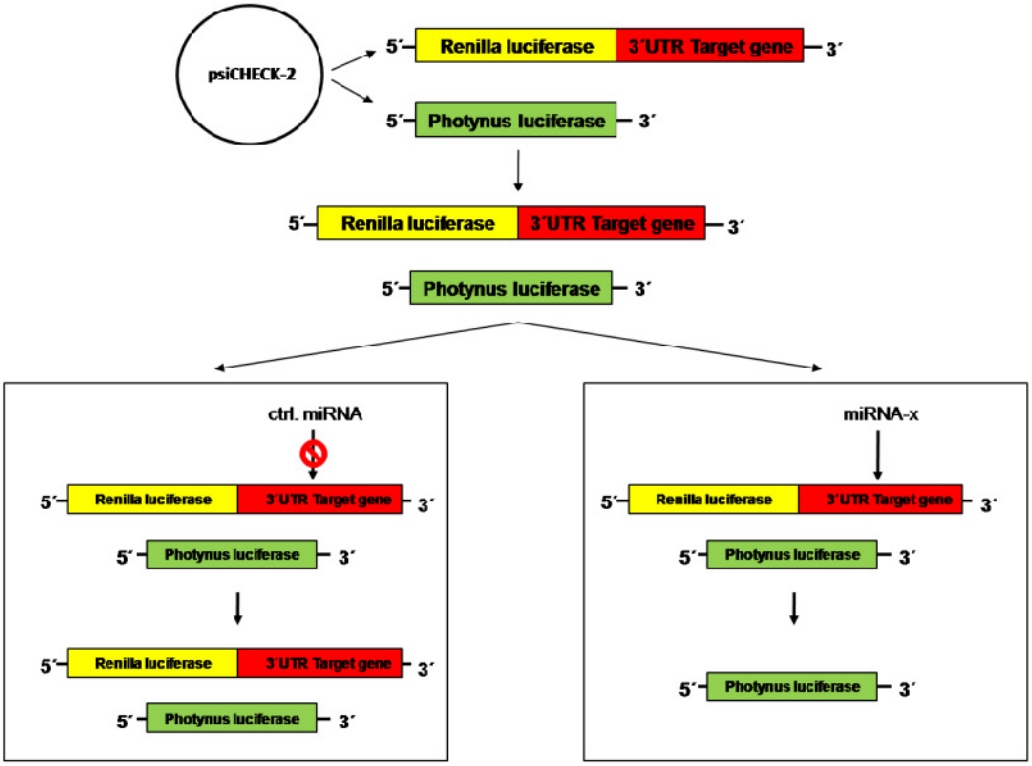
2.2.5 Sample preparation for pSILAC analysis

Changes in the proteome of miRNA-133b transfected HeLa cells were analyzed by performing a slightly modified version of the method described by Selbach *et. al* (Selbach et al, 2008) in cooperation with Dr. Bernd Thiede from the Biotechnology Centre of the University of Oslo (Oslo, Norway). Briefly, HeLa cells were cultivated during 5 passages in "light" culture medium (see 2.1.8) in order to ensure a complete labeling of the proteins with amino acids marked with light isotopes. "Light"-labeled cells were transfected for 8 hours with the 5 pmol control miRNA (ctrl. miRNA) or miRNA-133b. Subsequently, cells were transferred to culture medium containing medium-heavy or heavy isotope-labeled amino acids and pulse labeled for another 40 hours. After this time, both groups of cells were stimulated in the same media with 20 ng/ml TNFα for 6 hrs. Finally, stimulated labeled cells were collected by trypsination and sent out to our cooperation partners in Oslo for downstream mass spectrometry quantification of protein levels.

2.2.6 Molecular biological methods

2.2.6.1 Cloning of 3´-UTR luciferase reporter constructs

Validation of miRNA-targets with canonical miRNA binding sites was performed by cloning the complete 3'UTR of the predicted genes. To this end 3´-UTRs were amplified by polymerase chain reaction (PCR) using AccuPrime *Taq* Polymerase High Fidelity and primers designed to have restriction sites for XhoI and NotI (see 2.1.3, Table 3) PCR products were subcloned into pCR2.1-TOPO (Invitrogen) and subsequently cloned into XhoI/NotI digested psiCHECK-2 plasmid from (Promega). This vector enables monitoring of changes in expression of a target 3´-UTR fused to a reporter gene. It uses *Renilla* luciferase as primary reporter gene, and the 3´-UTR of interest is cloned into a multiple cloning region located downstream of the *Renilla* translational stop codon. Initiation of the RNAi process by synthetic miRNA or in vivo-expressed miRNAs results in down-regulation of the fusion mRNA. PsiCHECK-2 also contains a second reporter gene, *Photynus* (firefly) luciferase, which allows normalization of *Renilla* luciferase expression thus compensating for differences of the transfection efficiency of different samples (Scheme 9).

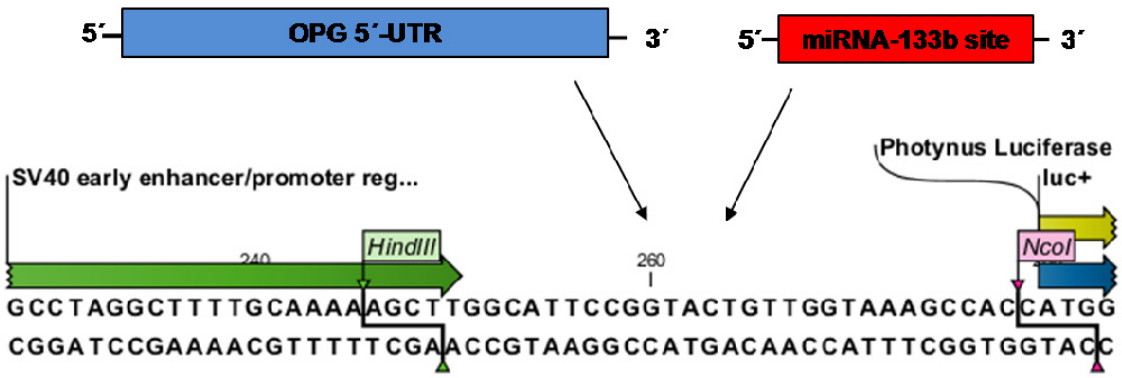


Scheme 9. Action mechanism of psiCHECK-2 3´UTR luciferase reporter constructs for miRNA target

2.2.6.2 Generation of 5´-UTR luciferase reporter constructs

Validation of potential binding sites in the 5´-UTR of OPG was performed by inserting either the complete 5´-UTR or the predicted miRNA-133b binding sequence only. Amplification of the 5´-UTR was performed by polymerase chain reaction (PCR) using AccuPrime *Taq* Polymerase High Fidelity and primers designed to have restriction sites for HindIII and NcoI (fw-primer AAGCTTTTTTTTTCCCCTGCTCTCC ; re-primer CCATGGTGTGGTCCCCGGAAACC). The PCR product was subcloned into pCR2.1-TOPO (Invitrogen) and subsequently inserted into HindIII/NcoI digested pGL3-ctrl plasmid (Promega) (Scheme 10). To generate a luciferase reporter plasmid containing only the *in-silico* predicted miRNA-133b binding sequence in the 5´-UTR of OPG, 5´-phosphorylated DNA oligos (MWG) corresponding to this sequence and harboring HindIII and NcoI restriction sites (fw-oligo P-AGCTAAGCCCCTGAGGTTTCCGGGGACCACT; re-oligo P-CATGAGTGGTCCCCGGAAACCTCAGGGGCTT) were annealed and ligated into HindIII/NcoI digested pGL3-ctrl plasmid. Oligo annealing was performed by dilution of equal molar amounts of both fw- and re-oligo in 1x oligo annealing buffer and heating this solution at 95°C for 5 min. Finally, the solution was allowed to cool slowly to room

temperature (RT) by unplugging the power supply of the heating block. Annealed oligos were stored at -20°C.



Scheme 10. Cloning strategy for generating 5´-UTR miRNA-targeting reporter constructs using pGL3-ctrl

2.2.6.3 RNA isolation and cDNA synthesis

Total RNA for quantitative PCR and Microarray analysis was isolated with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the total RNA-isolation protocol recommended by the manufacturer and using glycogen (Ambion, Austin, TX, USA) as a carrier for precipitation of nucleic acids. 2 µg total RNA per sample were transcribed to cDNA with random hexamer primers (Fermentas, Burlington, Canada) and SuperScript® II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) as per manufacturer´s instructions. After reverse transcription all samples were subjected to DNAseI (Invitrogen) treatment in order to remove contaminating genomic DNA.

2.2.6.4 Semi-quantitative real-time PCR

Analysis of gene expression was performed using gene-specific primers specifically designed to bind and amplify only mRNA sequences (see 2.1.4). Amplification and detection reactions were done using FasSYBR® Green master mix from Applied Biosystems (Carlsbad, CA, USA). Briefly, 4 µl diluted cDNA (1 to 50 aqueous dilution, see 2.2.7.1), 1.2 µl 1:1 primer mix (1.25 µM each) were added to 5 µl FastSYBR® Green master mix per well. Samples were measured in triplicates using a 7900HT fast real-time PCR system

(Applied Biosystems) set to perform the "fast" amplification protocol. Fold-changes in gene expression were calculated using the comparative ΔΔCt method and taking human acidic ribosomal protein (HuPO) as an internal standard (Bookout et al, 2006).

2.2.6.5 miRNA quantification

Quantitative PCR of miRNA was performed with TaqMan® miRNA assays from Applied Biosystems using 100 ng total RNA for the reverse transcription step. All samples were diluted 1 to 7.5 with ddH_2O and analyzed with a 7900HT fast real-time PCR system (Applied Biosystems). Fold-changes in gene expression were calculated using the comparative ΔΔCt method and RNA-U6B small nuclear (RNU6B) as internal standard (Bookout et al, 2006).

2.2.7 Biochemical methods

2.2.7.1 SDS-PAGE and Western blotting.

Protein lysates were generated by harvesting $5*10^5$ cells previously transfected and/or stimulated with apoptotic stimuli (see 2.2.8) in 100 µl 1x Laemmli sample buffer (Laemmli, 1970). 10-20 µl per lane of cell lysates were separated by SDS-PAGE. Tris-Glycine buffered SDS-polyacrylamide gels with 5% stacking gel and 12% resolving gel were used. Gels were run in 1x PAGE buffer at 100V for 60-90 min. Following SDS-PAGE proteins were transferred to PVDF membrane (Millipore, Billerica, MA, USA) using a semi dry electroblotting protocol. This was performed using a bloting machine and 1x Western blot buffer. Protein transfer took place at 1 mA/cm^2 for two hours. Following transfer the membrane was rinsed with TBS/T and blocked for one hour at RT in TBS/T containing 5% (v/v) non fat dry milk. The membrane was incubated overnight at 4°C on an orbital incubator with the primary antibody diluted in TBS/T containing 3% (v/v) non fat dry milk (see 2.1.2 Table 2). Next day, the antibody solution was removed and the membrane was rinsed at RT four times 15 min with fresh TBS/T. Horseradish peroxidase (HRP) -coupled secondary antibodies diluted in TBS/T (see 2.1.2 Table 2) was added to the membrane for one hour at RT under constant shaking. Following incubation, the membrane was washed three times 10 min. with fresh TBS/T. Enhanced chemoluminescence (ECL) reaction was performed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the

manufacturer´s instructions. Membranes were analyzed using LAS-3000 CCD imaging system from Fujifilm (Tokyo, Japan). Band intensity was quantified densitometrically using the AIDA Biopackage -2D (Raytest, Straubenhardt, Germany).

2.2.7.2 OPG ELISA.

Levels of secreted OPG were determined in the supernatant of transfected cells with the human Osteoprotegerin DuoSet from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's indications. To this end HeLa cells were seeded in 96-well plates, transfected with 5 pmol miRNA or/and 15 pmol antimiRNA (αmiRNA) and incubated for 48 or 72 hours in 300 µl culture medium before collection of the supernatants. At the specified time points 250 µl supernatants were collected and either used immediately for the determination of secreted OPG or stored at -80°C. All samples were analyzed in triplicates using a Spectramax 190 reader from Molecular Devices (Sunnyvale, CA, USA). Values were normalized to total number of metabolically active cells per well as determined by MTT-assay performed with the same culture plates (see 2.2.5.3).

2.2.7.3 MTT-Assay

A 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell survival and proliferation assay was used for normalizing the amount of secreted OPG, as determined by ELISA (see 2.2.5.2), to the total number of living cells per well (Mosmann, 1983). Briefly, MTT was diluted in PBS to a final concentration of 5 mg/ml, sterile filtered and stored away until use at -20°C. At the specified time points, MTT stock solution was further diluted with DMEM medium (w/o FCS) to a final concentration of 100 µg/ml. To perform the assay 50 µl of this solution were added to the culture plate containing the transfected cells in 50 µl supernatant (see 2.2.5.2). Treated cells were incubated for another 5 hours at 37°C and controlled hourly with a light microscope for the formation and precipitation of violet formazan crystals. Next, cell culture supernatants were removed carefully, taking care not to disturb the cell monolayer, and adherent cells were lysed with 100 µl DMSO. Finally, DMSO samples containing the diluted formazan crystals were transferred to ELISA plates and the OD_{570} values were determined by using a Spectramax 190 reader from Molecular Devices.

2.2.7.4 NF-κB activity luciferase assay

For determining NF-κB activity HeLa cells were co-transfected with 30 pmol miRNA and/or 90 pmol αmiRNA, 100 ng pGL3.ELAM.tk and 10 ng pRL-SV40 (see 2.2.2). Transfected cells were incubated for 48 hrs. before they were either left untreated or primed for 4 hrs. with 20 ng/ml TNFα or 10 µg/ml poly(I:C). Luciferase activity was measured using the Dual-Luciferase® Reporter Assay System from Promega (Madison, WI, USA) as per manufacturer´s recommendations. Briefly, transfected stimulated cells were washed once with PBS and lysed with 200 µl of 1x passive lysis buffer (PLB). Cell lysates were incubated on an orbital plate shaker at 200 rpm for 15 min. in order to assure complete lysis of the cells. Next, samples were diluted 1 to 10 using PLB. Luciferase assays were performed in triplicates in white 96-well plates using 15 µl lysate and 35 µl of both substrates for the Photynus and Renilla luciferases per well. Measurement of light emission was performed on Victor luminometer (Perkin Elmer, Waltham, MA, USA). Cells transfected with ctrl. miRNA were used as reference point for each different type of treatment.

2.2.7.5 miRNA-targeting luciferase assay

Luciferase reporter assays were performed by co-transfecting HeLa cells with 100 ng psiCHECK-2 3´-UTR or pGL3-ctrl 5´-UTR constructs and 5 pmol ctrl. miRNA or miRNA-133b. For blocking experiments 15 pmol ctrl. αmiRNA or αmiRNA-133b were included. In 5´-UTR targeting experiments pRL-SV40 was included as normalization control. 48 hours post-transfection cells were rinsed once with PBS and lysed with PLB. Cell lysates were incubated on an orbital plate shaker at 200 rpm for 15 min. in order to assure complete lysis. Next, samples were diluted 1 to 10 using PLB. Dual luciferase assay was performed in triplicates on white 96-well plates using 15 µl lysate and 35 µl of both substrates for the *Photynus* and *Renilla* luciferases per well. Measurement of light emission was performed on VICTOR luminometer (Perkin Elmer, Waltham, MA, USA). Total luciferase activity (*Renilla reniformis)* was calculated by normalizing to the firefly *(Photinus pyralis)* luciferase in order to correct for differences in the transfection efficiency.

2.2.8 Apoptosis detection

Samples for apoptosis quantification were prepared as follows: HeLa cells were transfected with 5 pmol ctrl. miRNA or miRNA-133b. For inhibition experiments 15 pmol ctrl. αmiRNA or αmiRNA-133b were co-transfected. 48 hours post-transfection cells were stimulated with 20 ng/ml TNFα (with or without 10 µg/ml cycloheximide (CHX)), 100 ng/ml of a cross-linking activating antiCD95 antibody (αCD95) or 20 ng/ml recombinant human TRAIL (rhTRAIL). 1µM staurosporine (STA) was used as a positive control. Chemical inhibition of apoptosis was achieved by incubating the cells with 50 µM carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone (Z-VAD-FMK) one hour prior to addition of DR ligands or chemical apoptosis inducers. Four hours after stimulation cells were assessed for the presence of activated caspases, poly (ADP ribose) polymerase (PARP) cleavage, genomic fragmentation or loss of membrane integrity.

2.2.8.1 Quantification of active CASP8 and 3

Apoptotic cells were quantified by flow cytometric determination of CASP8 and 3 activation using fluorescent-labeled inhibitors of caspases (FLICA^{TM}) from Immunochemistry Technologies (Bloomington, MN, USA). All procedures were done according to manufacturer´s instructions.

2.2.8.2 TUNEL assay

Genomic DNA fragmentation was assayed with the ApoDirect® kit from BD-Biosciences which is a two-color staining method for labeling DNA breaks and total cellular DNA to detect apoptotic cells by flow cytometry. Staining of the cells was performed according to the instructions delivered by the manufacturer.

2.2.8.3 PI-incorporation assay

Propidium iodide (PI) incorporation was used for determining the overall cell vitality. Briefly, trypsinized cells were washed once with warm PBS and resuspended in FLICA washing buffer containing 20 µg/ml PI. Cells were incubated for 30 min. at RT protected from light before flow cytometry quantification.

2.2.9 Statistics

Unless otherwise specified, data shown is representative for the results of at least three independent experiments. Statistical significance was calculated by two-tailed Student's *t*-test and $p<0.05$ was considered significant.

3. Results

3.1 miRNA expression profile of mycobacterial infection

Based on the importance of miRNAs for the mounting and appropriate function of innate immune responses, a miRNA expression profile of human macrophages infected with *M. tuberculosis* H37Rv (virulent) or *M. bovis* BCG (avirulent) was generated. PMA-differentiated THP-1 cells were infected and incubated for 24 or 48 hrs. At the respective time points, total RNA was isolated and analyzed on miRNA expression microarrays. Of the 534 human miRNAs covered by the scanning platform only nine were differentially regulated (fold-change ≤ -1.5 or ≥ 1.5) after infection of macrophages with the mycobacteria (Table 1.).

Table 9. miRNAs differentially expressed in THP1 macrophages after mycobacterial infection

	Name	*M. bovis* BCG		*M. tuberculosis* H37Rv	
		FCH 24[1]	FCH 48[2]	FCH 24	FCH 48
G1	miRNA-133b	52.5	2.2	n.d[3].	n.d.
	miRNA-146a	3.4	2.7	n.d.	n.d.
	miRNA-155	2.3	2.5	1.5	1.5
	miRNA-339 (G4)	1.6	-2.4	-2.4	-2.2
G2	miRNA-137	-7.9	-2.8	n.d.	n.d
	miRNA-145	-4	-3.9	n.d	n.d.
	miRNA-340	-2	-4.7	n.d.	n.d.
G3	let-7e (G4)	-2.1	1.6	-1.7	-2
	miRNA-27a (G4)	2.7	-3	-1.6	-2.1

[1] FCH 24 = fold change in miRNA expression after 24 hours infection.
[2] FCH 48 = fold change in miRNA expression after 48 hours infection.
[3] n.d. = not detected

According to their expression profile the detected miRNAs could be categorized in four different groups. The first group (G1) contained miRNAs showing consistent up-regulation during the complete time course of the experiment after infection with *M. tuberculosis* H37Rv, *M. bovis* BCG or in both cases. These included previously well characterized immunoregulators like miRNA-146a and -155, but also novel ones such as miRNA-133b and -339 (O'Connell et al, 2007; Taganov et al, 2007). The second category (G2) of miRNAs included those detected as down-regulated at both time points after

infection with *M. bovis* BCG, such as miRNA-137, -145 and -340. MiRNA-27a and let-7e fitted in the third group (G3) and were characterized by a more dynamic expression profile and opposite regulation polarities at 24 and 48 hrs. of infection with *M. tuberculosis* H37Rv or *M. bovis* BCG. Finally, the fourth group (G4) of miRNAs enclosed those displaying contradictory expression profiles depending on the time point and whether the cells were infected with *M. tuberculosis* H37Rv or *M. bovis* BCG. For all miRNAs, except miRNA-137 the expression profile delivered by the microarray analysis could be corroborated by quantitative polymerase-chain-reaction (qPCR) using independent samples. In the case of *M. tuberculosis* H37Rv infection, contrary to the results delivered by the microarray experiments, the qPCR analysis revealed a previously undetected differential regulation of the miRNAs -133b, -137, -145, 146a and -340 (Figure 1).

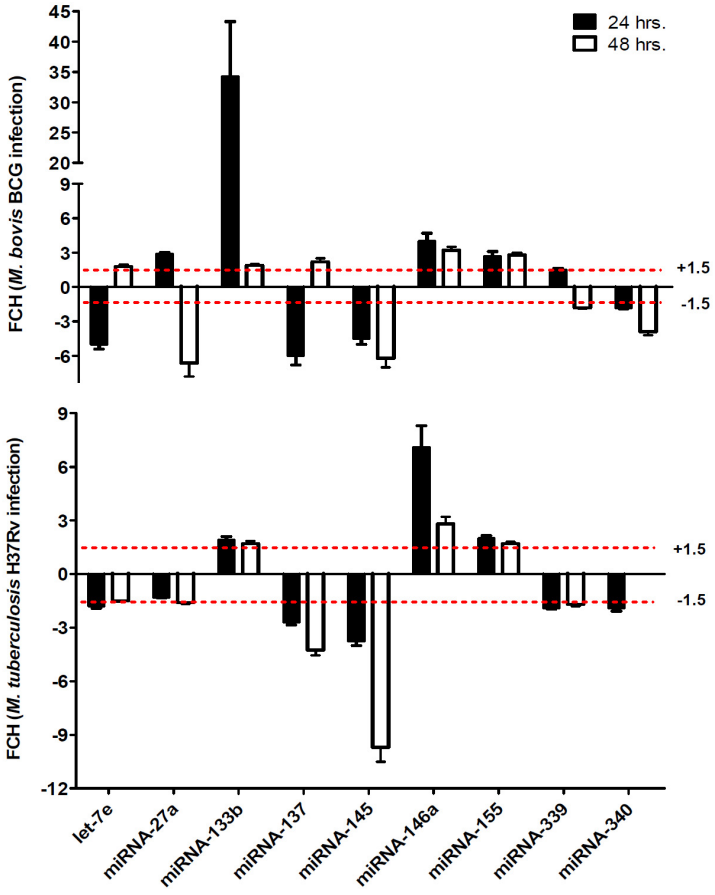


Figure 1. qPCR validation of miRNA microarray expression profile.

PMA-differentiated THP-1 cells were infected with *M. bovis* BCG (upper graph) or *M. tuberculosis* H37Rv (lower graph) and incubated for 24 (black bars) and 48 hrs (white bars). At each time point, total RNA was harvested and used for miRNA-specific qPCR analysis. Infection samples were compared to differentiated uninfected cells that had been cultivated simultaneously for the same period of time. RNU6B was used as an internal standard for normalization. Values were calculated by performing the ΔΔCt-method (Bookout et al, 2006).

In summary, using this platform very strong effects such as up-regulation of miRNA-133b in *M. bovis* BCG infected cells, mild effects like those observed for miRNA-339 and significant down-regulation of miRNA species such as the one observed for miRNA-145 and -137 could be detected. These results indicate that mycobacterial infection of human macrophages leads to changes in miRNA expression levels.

3.2 miRNA-133b sensitizes resistant cells to TNFα-induced apoptosis

Next to the identification of miRNAs differentially regulated after infection of human macrophages with mycobacteria, their involvement in the regulation of innate immunity was characterized. As a special focus the investigation of the role these miRNAs might play in the regulation of TNFα-signaling and TNFα-induced apoptosis was chosen. The major challenges of working with miRNAs and macrophages are the engineering, transfection and over-expression of genetic constructs (Lee & Reiner, 2009; Zhang et al, 2009). Therefore, to be able to easily test all candidate miRNAs, a well characterized and easy to transfect HeLa cell line model was selected. HeLa cells are TNFα-responsive but, due to strong activation of the NF-κB signaling pathway, they do not undergo apoptosis. Several years ago it was demonstrated that the pro-apoptotic effect of TNFα could be blocked in a NF-κB dependent manner (Beg & Baltimore, 1996; Liu et al, 1996b; Van Antwerp et al, 1996; Wang et al, 1996). Upon activation, NF-κB is released, translocates to the nucleus and induces the expression of anti-apoptotic molecules such as the cIAP1/2, XIAP, (cFLIP), manganese superoxide dismutase (MnSOD), TNF-receptor associated factor 1 and 2 (TRAF1/2) and several members of the BCL-2 family (Dutta et al, 2006; Shen & Tergaonkar, 2009).

In order to test whether the identified miRNAs play regulatory functions within the TNFα-signaling pathway, HeLa cells were transfected with synthetic miRNA precursors that mimicked the differentially regulated miRNAs detected during the infection screen. As a negative control the cells were transfected with scrambled miRNA (ctrl. miRNA), a molecule with no known sequence homology to any human gene. 48 hrs. later the cells were stimulated with TNFα and, the activation status of pro-apoptotic CASP8 was measured. Cycloheximide (CHX), a potent protein synthesis inhibitor, was added during the stimulation in order to block the *de-novo* synthesis of anti-apoptotic proteins and force the cells to undergo programmed cell death. Irrespective of the transfected miRNA, unstimulated cells showed

low levels (4 to 8% of the cells) of cleaved CASP8. Four hours after addition of TNFα and CHX to the cell culture 40, 35, 28 and 42% of the cells respectively transfected with the negative control, miRNA-27a, -146a or -155, stained positive for active CASP8. This proportion increased to 75% of the cells after transfection of miRNA-133b, indicating that this miRNA might be pro-apoptotic (Figure 2).

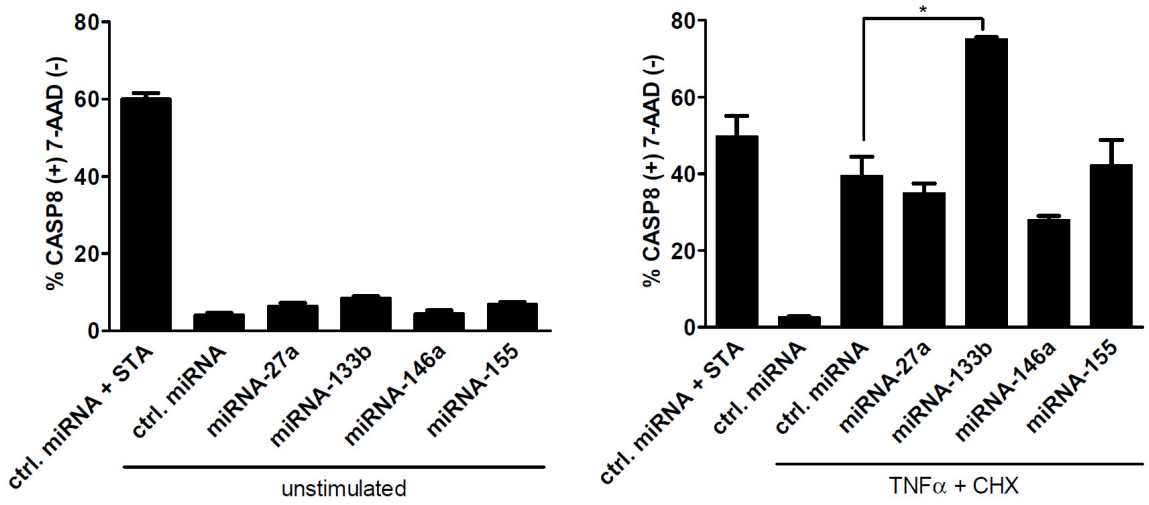


Figure 2. miRNA-133b enhances CASP8 cleavage in HeLa cells treated with TNFα and CHX.

Cells were transfected with synthetic miRNA mimics or a negative control (ctrl. miRNA). After incubation for 48 hrs., cells were either left untreated (left) or stimulated with 20 ng/ml TNFα and 10 µg/ml CHX in order to induce apoptosis (right). Four hours after stimulation the percentage of cells displaying the activated form of the pro-apoptotic CASP8 was determined by flow cytometry.7-AAD was included as an exclusion marker for cells with a leaky plasma membrane(Schmid et al, 1992). Cells transfected with ctrl. miRNA were treated with staurosporine (STA), a potent pro-apoptotic substance, as a positive control. Both graphs are representative of at least three independent experiments.* symbolizes a p-value smaller than 0.01. Errors bars = standard deviation

Defining apoptosis based on quantification of only one characteristic feature, for instance the activation of pro-apoptotic caspases, is insufficient and increases the risk of focusing on experimental artifacts (Kroemer et al, 2009). In order to avoid this, the response of miRNA-133b transfected cells to TNFα and CHX was further characterized by quantifying the degree of genomic DNA fragmentation, a typical feature of apoptotic cells (Wyllie et al, 1984). First dUTP nick end labeling (TUNEL) assay was performed with cells that had been transfected with either the negative control or miRNA-133b and subsequently incubated for another 48 hrs (Gavrieli et al, 1992). Both groups of transfected cells showed almost no detectable signs (~2% positive cells) of genomic DNA disintegration (Figure 3A). After stimulation with TNFα and CHX, 20% of the ctrl. miRNA transfectants stained positive for dUTP-FITC. This proportion increased to 54% of the cells in the case of miRNA-133b,

representing a 2.7 fold increase with respect to the result observed for cells transfected with the negative control (Figure 3B).

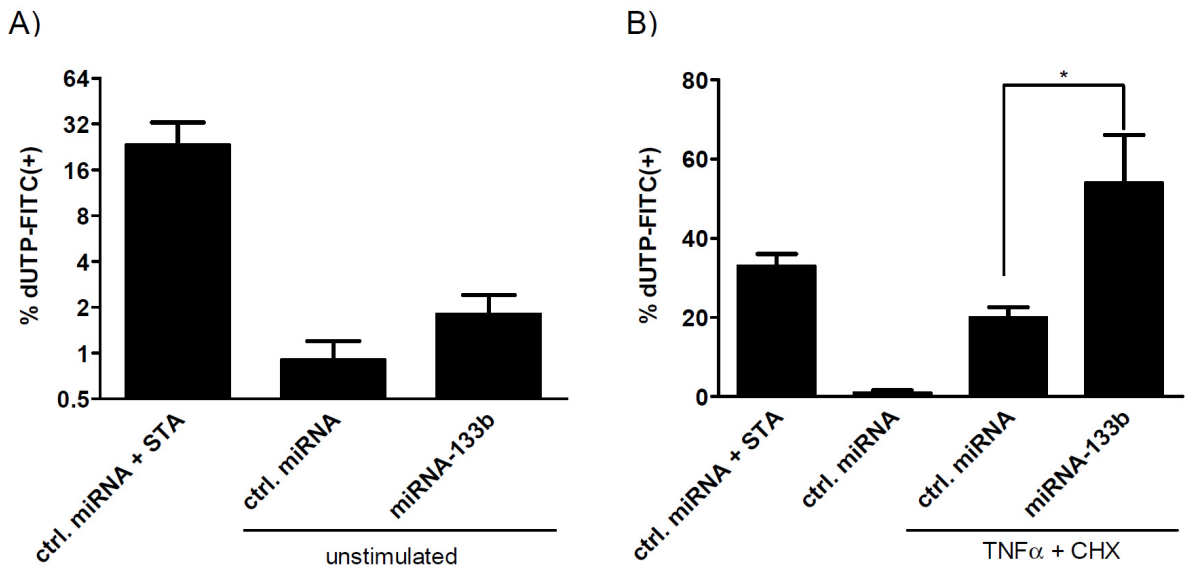


Figure 3. miRNA-133b transfectants undergo enhanced genomic DNA fragmentation after treatment with TNFα and CHX.

Cells were transfected with a ctrl. miRNA or miRNA-133b mimic and incubated for 48 hrs. After incubation, transfected cells were either left untreated (A) or stimulated with 20 ng/ml TNFα and 10 µg/ml CHX in order to induce apoptosis (B). Four hours after stimulation all cells were harvested and stained for genomic double breaks using the TUNEL assay. STA treated cells served as positive control. Graphs are representative of at least three independent experiments. * symbolizes a p-value smaller than 0.01. Errors bars = standard deviation

After observation of increased mortality mediated by miRNA-133b, the next step was to characterize its pro-apoptotic potential in the absence of CHX. MiRNA-133b treatment sensitized otherwise resistant HeLa cells to TNFα-triggered CASP8 and 3 activation (Figure 4A). In line with this, poly (ADP ribose) polymerase (PARP) cleavage, another hallmark of apoptotic cells, could only be observed in miRNA-133b transfectants (Figure 4B) (Kaufmann et al, 1993). Both effects were sequence specific, since transfection of the scrambled negative control did not result in an altered activation status of CASP8, 3 or PARP degradation. Moreover, both activation of pro-apoptotic caspases and cleavage of PARP could be fully restrained by adding a specific miRNA-133b inhibitor (αmiRNA-133b), but not the negative control (ctrl. αmiRNA). The basal activation status of CASP8 and 3 in unstimulated transfected cells, as well as the amount of cleaved PARP, was slightly elevated in miRNA-133b transfected samples (Figure 4A and B). Although these effects were small they were significant and specific to this miRNA. In summary, these results suggested that miRNA-133b has a pro-apoptotic nature capable of impairing the pro-survival program characteristic

of TNFα-treated cells. Furthermore, this implied a down-regulation of anti-apoptotic molecules.

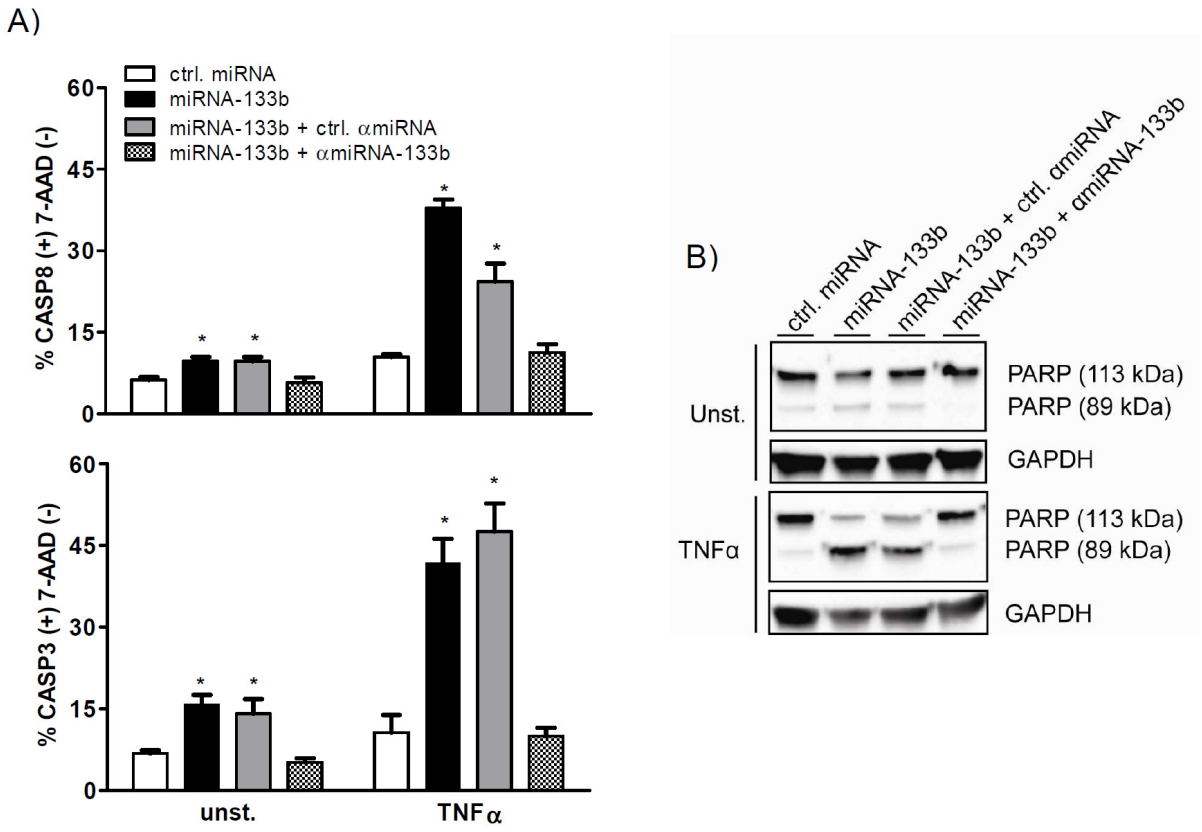


Figure 4. TNFα-resistant cells become sensitive after miRNA-133b transfection.

Cells were transfected with miRNA-133b alone or together with a control antimiRNA (ctrl. αmiRNA) or a specific miRNA-133b inhibitor (αmiRNA-133b). After 48 hrs. cells were either left untreated or stimulated four hours with 20 ng/ml TNFα. **A)** Treated cells were harvested, stained and scanned by flow cytometry for the presence of cleaved active CASP8 (upper graph) and 3 (lower graph). Cells transfected with ctrl. miRNA alone were used as a reference. **B)** Western blot analysis of PARP in transfected, unstimulated (upper panel) and TNFα-treated cells (lower panel). GAPDH was used as an internal loading control. Graphs are representative of at least three independent experiments. * symbolizes a p-value smaller than 0.01. Errors bars = standard deviation

3.3 miRNA-133b promotes FasL-triggered apoptosis and leads to exacerbated TRAIL responsiveness

HeLa cells are mostly Fas ligand (FasL) refractory and do not undergo apoptosis upon receptor ligation. In a fashion similar to the mechanism responsible for the TNFα-resistance, Fas (CD95) stimulation in these cells leads to strong NF-κB activation and *de-novo* synthesis of potent anti-apoptotic molecules (Wajant et al, 2003). To test whether miRNA-133b is capable of modifying the cellular response to additional members of the DR ligand family, HeLa cells were transfected with miRNA-133b, incubated for 48 hrs. and activated either

with a cross-linking antibody against Fas or with recombinant human TRAIL (rhTRAIL). After stimulation, the activation status of the cell was assessed by quantification of active CASP8, 3 and PARP cleavage.

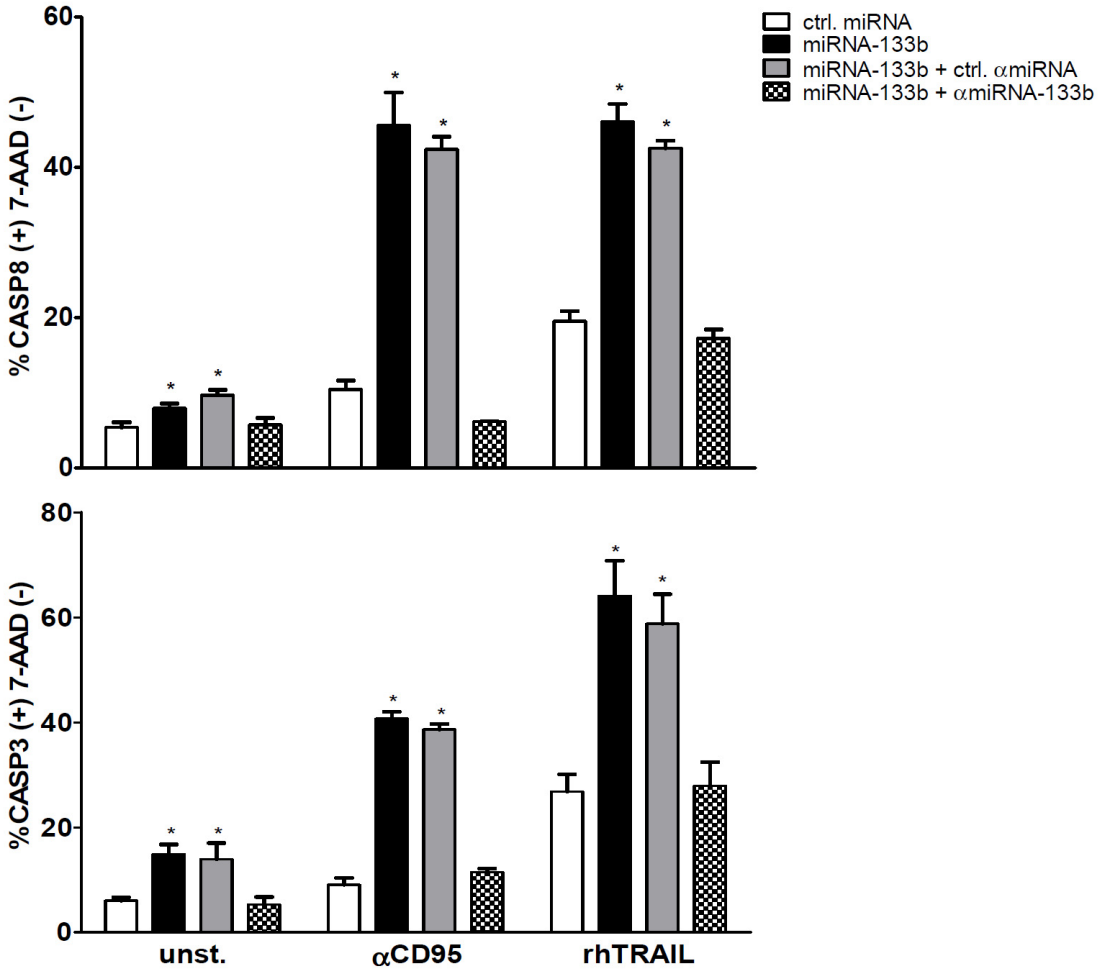


Figure 5. miRNA-133b sensitizes HeLa cells to FasL and TRAIL induced caspase activation.

Cells were transfected with miRNA-133b alone or together with a control antimiRNA (ctrl. αmiRNA) or a specific miRNA-133b inhibitor (αmiRNA-133b). After 48 hrs. cells were left untreated or stimulated four hours with either 100 ng/ml of a cross-linking activating antiCD95 antibody (αCD95) or 20 ng/ml recombinant human TRAIL (rhTRAIL). Treated cells were harvested, stained and scanned by flow cytometry for the presence of cleaved active CASP8 (upper graph) and 3 (lower graph). Cells transfected with ctrl. miRNA alone were used as a reference. Graphs are representative of at least three independent experiments. * symbolizes a p-value smaller than 0.01. Errors bars = standard deviation

Transfection of the cells with miRNA-133b impaired the cellular obstruction of caspase activation after Fas stimulation, as reflected by fivefold higher percentage of cells harboring the cleaved active form of CASP8 and 3 (compared to the ctrl. miRNA) (Figure 5). In line with this, PARP cleavage was only observed in stimulated cells previously transfected with the miRNA-133b (Figure 6). TRAIL stimulated cells, were characterized by a strong

pro-apoptotic phenotype. Transfection of miRNA-133b enhanced this natural responsiveness and led to an exacerbated apoptotic response. After 4 hrs. TRAIL stimulation, 20 and 25% of the ctrl. miRNA cells stained positively for CASP8 and 3, respectively. This proportion expanded to 46 and 64% in miRNA-133b transfected cells (Figure 5). The increased levels of initiator and executor caspases correlated well with an enhanced cleavage of PARP, as demonstrated by the almost complete absence of the full-length protein after transfection of the cells with miRNA-133b and TRAIL stimulation (Figure 6). In all cases the observed effects were sequence specific and could only be fully blocked by co-transfection of a specific αmiRNA, but not by an unspecific one. Hence, these results demonstrate that the pro-apoptotic potential of miRNA-133b was not restricted to TNFα-treatment and that it also affected in a specific manner the cellular responses to FasL or TRAIL.

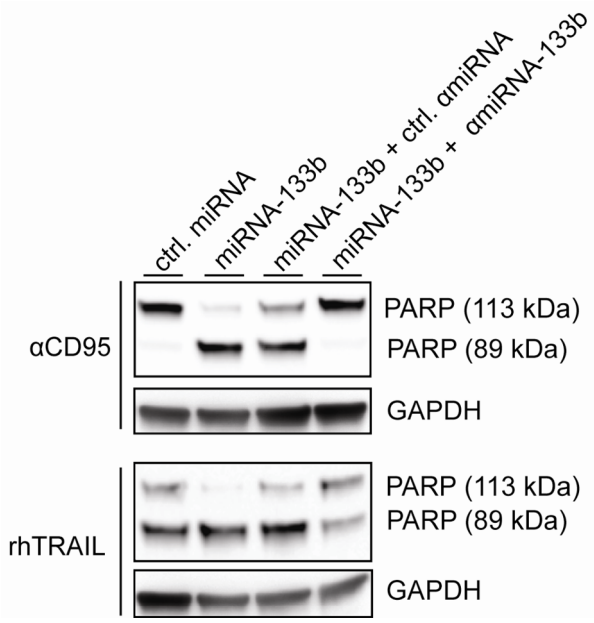


Figure 6. miRNA-133b sensitizes HeLa cells to FasL and TRAIL induced PARP cleavage.

Cells were transfected with miRNA-133b alone or together with a control antimiRNA (ctrl. αmiRNA) or a specific miRNA-133b inhibitor (αmiRNA-133b). After 48 hrs. cells were stimulated four hours with either 100 ng/ml of a cross-linking activating antiCD95 antibody (αCD95) or 20 ng/ml recombinant human TRAIL (rhTRAIL). After stimulation, cell protein lysates were prepared and the proteolytic cleavage of PARP was assessed by Western blot. GAPDH was used as an internal loading control. Blots are representative of at least three independent experiments.

3.4 Loss of plasma membrane integrity is enhanced by miRNA-133b

Late apoptotic cells are characterized by compromised plasma membrane integrity (Gorczyca, 1999; Patel et al, 2006). To test whether miRNA-133b led to an altered permeability status or promiscuous rupture of the cellular envelope after cell death induction,

transfected HeLa cells were stimulated with different DR ligands and stained with propidium iodide (PI). PI is membrane impermeant and generally excluded from viable cells. It is commonly used for identifying cells with impaired membrane integrity. In average 20.3% of the unstimulated cells transfected with miRNA-133b were PI-positive, compared to ctrl. miRNA transfected cells. Addition of αmiRNA-133b decreased this proportion to 6.4% (Figure 7; upper row). This effect of miRNA-133b on the cell membrane permeability became more evident after stimulation of the cells with DR ligands, as demonstrated by increased PI incorporation. Treatment with TNFα, αCD95 or rhTRAIL resulted in 57, 77 and 63% PI-positive cells, respectively. Once more, this effect was specific for miRNA-133b, as it could be blocked exclusively by a sequence specific inhibitor but not by a negative control sequence (Figure 7, lower three rows).

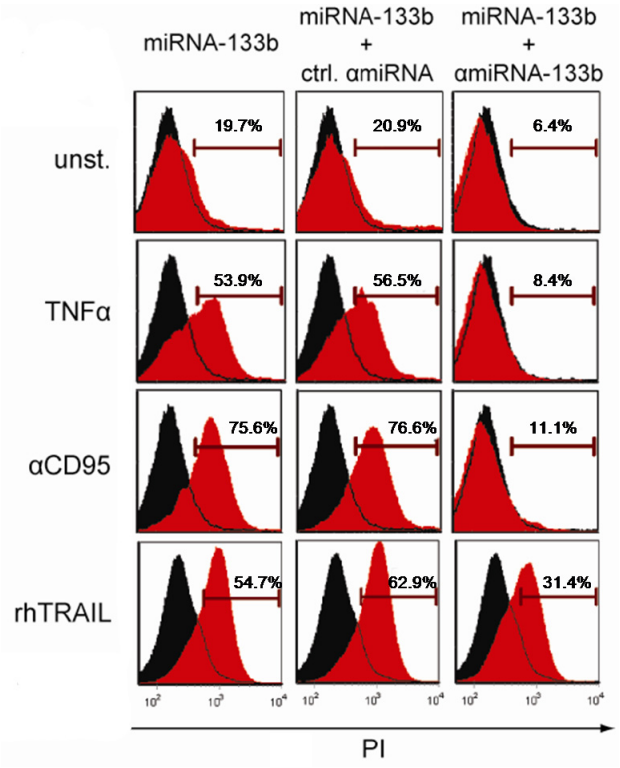


Figure 7. miRNA-133b treatment results in enhanced loss of membrane integrity.

Cells were transfected with miRNA-133b alone or together with a control antimiRNA (ctrl. αmiRNA) or a specific miRNA-133b inhibitor (αmiRNA-133b). After 48 hrs. cells were left untreated or stimulated four hours with either 20 ng/ml TNFα, 100 ng/ml of a cross-linking activating antiCD95 antibody (αCD95) or 20 ng/ml recombinant human TRAIL (rhTRAIL). Following stimulation both adherent and suspension cells were collected, stained with propidium iodide (PI) and analyzed by flow cytometry. Samples were compared to equally treated ctrl. miRNA transfected cells (black histogram). Results are representative for at least three independent experiments.

Caspases are a group of cysteine proteases critical for apoptosis of eukarytic cells (Wang & Lenardo, 2000). To confirm the apoptotic nature of the miRNA-133b effect on the

enhanced loss of membrane integrity, transfected HeLa cells were stimulated with DR ligands both in the absence or presence of carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK), a cell-permeable inhibitor that irreversibly binds to the catalytic site of caspases thus inhibiting the induction of apoptosis. Addition of Z-VAD-FMK to both un- and stimulated miRNA-133b transfected cells was characterized by a strongly reduced population of PI-incorporating cells, when compared to equally stimulated cells treated with the inhibitor solubilizing agent (Figure 8). Hence, miRNA-133b treatment of HeLa cells led to increased loss of membrane integrity in a caspase-dependent manner. This suggested that miRNA-133b as pro-apoptotic molecule is capable of impairing the mechanisms responsible for keeping an intact cellular envelope.

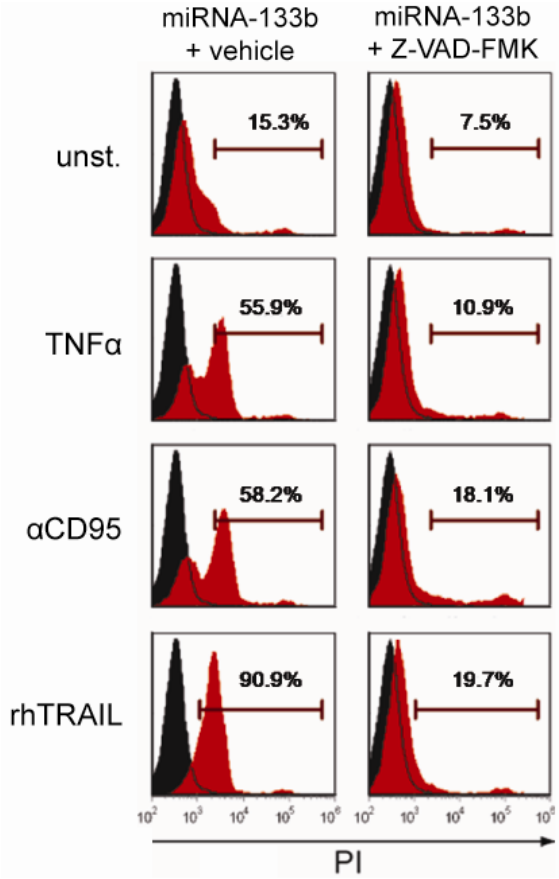


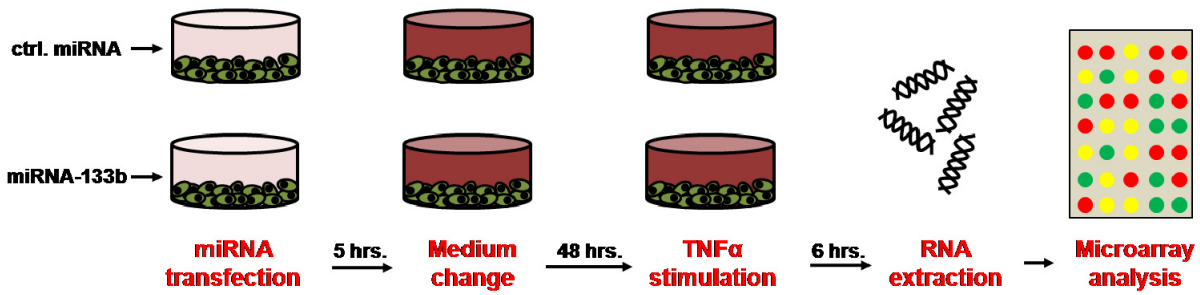
Figure 8. Loss of membrane integrity mediated by miRNA-133b is caspase dependent.

Cells were transfected either with a control miRNA or miRNA-133b. 48 hrs. after transfection cells were left untreated or stimulated four hours with either 20 ng/ml TNFα, 100 ng/ml of a cross-linking activating antiCD95 antibody (αCD95) or 20 ng/ml recombinant human TRAIL (rhTRAIL). Z-VAD-FMK (50 µM) or 0.01% DMSO (vehicle) were added simultaneously to the cells. After stimulation both adherent and suspension cells were collected, stained with propidium iodide (PI) and analyzed by flow cytometry. Samples were compared to equally treated ctrl. miRNA transfected cells (black histogram). Results are representative for at least three independent experiments.

3.5 miRNA-133b target identification

3.5.1 Microarray and pSILAC analysis of mRNA and protein expression

Following the determination and description of the pro-apoptotic nature of miRNA-133b, the question concerning the target genes and their involvement in the process of apoptosis sensitization was approached. Given the fact that miRNA regulation may be executed at the mRNA (mRNA slicing) or protein level (translational arrest), a dual target discovery strategy was used. First, taking advantage of the clear cut phenotype observed in HeLa cells, anti-apoptotic genes being regulated at the mRNA-level were characterized. Whole genome microarray expression-analysis allowed the identification of mRNAs with impaired expression after miRNA-133 transfection. Pursuing the idea that miRNA-133b might primarily act by restraining the induction of canonical anti-apoptotic factors, transfected cells were stimulated with TNFα for 6 hours prior to RNA collection (Scheme 11).



Scheme 11. Microarray gene expression analysis of TNFα-stimulated ctrl. and miRNA-133b transfected cells

Under these conditions a total of 213 genes emerged as down-regulated (FCH<-1.5; p-value<0.001). Consistent with results published by others the observed mRNA expression changes were not drastic and ranged between -1.5 and a minimum of -4.8 fold (Lim et al, 2005). In order to categorize the obtained results, the list of potential target genes was analyzed and filtered according to two criteria: 1) *in-silico* sequence analysis for the presence of potential miRNA-133b binding sites 2) known pro- or/and anti-apoptotic features. For the first filter the prediction databases TargetScan and MicroCosm Targets were used (Friedman et al, 2009; Griffiths-Jones et al, 2008; Grimson et al, 2007; Lewis et al, 2005). The second filter was achieved by performing a gene functional classification using the database for annotation, visualization and integrated discovery (DAVID) (Dennis et al, 2003; Huang da et al, 2009a; Huang da et al, 2009b).

According to this filtering strategy 13 genes with the ability to regulate apoptotic responses were identified. Eight of these were predicted to harbor miRNA-133b binding sites in their 3´-UTR (Table 10). Of all 13 genes the pro-apoptotic ones were BCL2/adenovirus E1B 19kD-interacting protein 3-like (BNIP3L), Killer cell lectin-like receptor subfamily K member 1 (KLRK1), sulfatase 1 (SULF1), sphingosine-1-phosphate phosphatase 1 (SGPP1), cytoplasmic FMR1 interacting protein 2 (CYFIP2), growth arrest-specific gene-1 (GAS1), forkhead box L2 (FOXL2) and PHD finger protein 17 (PHF17). On the other hand, the list of anti-apoptotic genes consisted of OPG, FAIM, FASN and GSTP1. The latter group represented the most interesting set of target gene candidates, since miRNA-133b is an apoptosis promoting, but not repressing molecule.

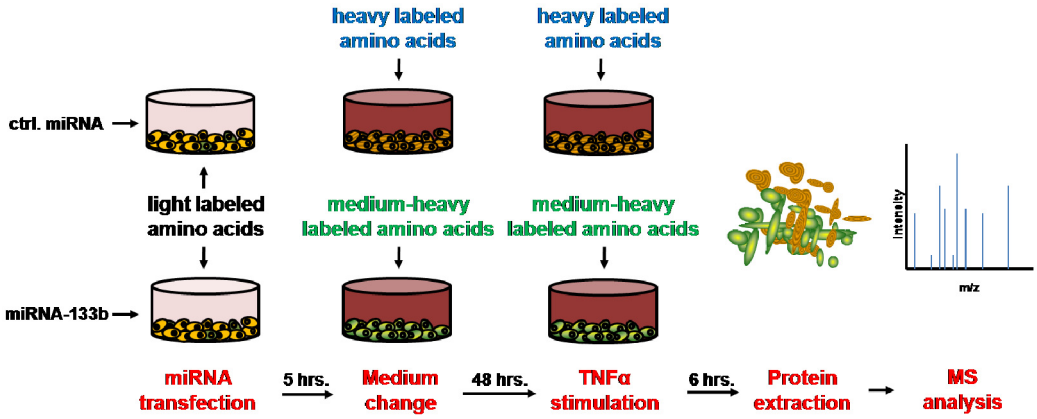
Table 10. Apoptosis regulatory genes down-regulated at the mRNA level in miRNA-133b transfected TNFα-stimulated HeLa cells

Accesion	Gene name	[1]FCH	Function	[2]Predicted miRNA-133b Target
NM_002546	**OPG**	**-2.9**	**anti-apoptotic**	**no**
NM_004331	BNIP3L	-2.4	pro/anti-apoptotic	yes
NM_007360	KLRK1	-2.3	pro-apoptotic	yes
NM_015170	SULF1	-2.2	pro-apoptotic	yes
NM_018147	**FAIM**	**-2.2**	**anti-apoptotic**	**yes**
NM_004104	**FASN**	**-2.1**	**anti-apoptotic**	**no**
NM_000852	**GSTP1**	**-2.0**	**anti-apoptotic**	**yes**
NM_030791	SGPP1	-1.9	pro-apoptotic	no
NM_014376	CYFIP2	-1.9	pro-apoptotic	no
NM_002048	GAS1	-1.8	pro-apoptotic	no
NM_023067	FOXL2	-1.7	pro-apoptotic	yes
NM_024900	PHF17	-1.7	pro-apoptotic	yes

[1] compared to ctrl. miRNA tranfected and TNFα-stimulated cells. p-value < 0.01
[2] As predicted by either TargetScan or MicroCosm Targets

As the second step of the target identification strategy and to complement the microarray analysis, a global protein profile of miRNA-133b transfected cells was generated. To this end the recently developed pulsed stable isotope labeling by amino acids in cell culture (pSILAC) approach was used (Selbach et al, 2008). Briefly, pSILAC is based on the labeling of cells with amino acids of dissimilar molecular mass. Once labeled, the cells are

transfected with the miRNA of interest or a negative control and both protein samples are analyzed and compared by mass spectrometry. This allows the exact identification and quantification of the protein content present in each sample. By direct comparison of both samples it is possible to identify proteins whose expression is repressed in the sample transfected with the miRNA of interest. Such proteins constitute very interesting miRNA target candidates, since the diminished expression level could be a direct consequence of the miRNA treatment. In this particular case analysis of HeLa cells transfected with miRNA-133b or the control miRNA was performed. Acting on the same assumption as for the microarray expression analysis, miRNA-133b and ctrl. miRNA transfected cells were also stimulated with TNFα for 6 hours prior to protein lysate harvest (Scheme 12).



Scheme 12. pSILAC analysis of TNFα-stimulated ctrl. and miRNA-133b transfected cells. Based on (Selbach et al, 2008)

Consistent with previous reports miRNA-133b transfection led only to overall mild effects on protein synthesis (Baek et al, 2008; Selbach et al, 2008). A total of 1480 proteins could be identified by mass spectrometry. Of these 25 displayed significant repression in the sample transfected with miRNA-133b and TNFα (Table 11). 19 proteins were predicted to comprise potential miRNA-133b binding sites, but only 6 correlated positively with the results obtained by microarray analysis (Table 10.). Among these, FASN and GSTP1 constituted the most interesting candidates due to their anti-apoptotic roles and relative strong repression at the protein level (2.6 and 2.1 respectively).

Table 11. Apoptosis regulatory proteins down-regulated in miRNA-133b transfected TNFα-stimulated HeLa cells

UniProt Name	[1]FCH repression	Positive correlation with microarray analysis	[2]Predicted miRNA-133b Target
TAGLN2	4.2	yes	yes
VIM	3.9	no	no
MYH9	2.7	yes	yes
FASN	**2.6**	**yes**	**no**
MYL12B	2.7	no	yes
CKAP4	2.3	yes	yes
NP	2.2	no	yes
PTBP1	2.2	yes	yes
GSTP1	**2.1**	**yes**	**yes**
MYL6	2.1	no	no
MSN	2.1	no	yes
SAE1	2	no	no
CPNE3	1.9	yes	yes
FSCN1	1.9	no	yes
CMPK1	1.8	no	yes
ARHGDIA	1.8	no	yes
CAP1	1.7	no	yes
MAT2B	1.7	no	yes
CARBP2	1.6	no	no
HMGCS1	1.6	no	no
IDH1	1.6	no	yes
NQO1	1.6	no	yes
RAB5C	1.6	no	yes
SERPINH1	1.5	no	yes

[1] Fold-change
[2] As predicted by either TargetScan or MicroCosm Targets

3.5.2 Target validation

Based on the results delivered by microarray and pSILAC analyses, selected genes were chosen for further validation and characterization. These included the anti-apoptotic genes FAIM, OPG, FASN and GSTP1. For the miRNA target validation process an experimental strategy covering three different aspects was used. These were: i) *in-silico* gene sequence analysis and prediction of potential miRNA-133b binding regions ii) Experimental demonstration of the direct physical interaction between the miRNA and the predicted target sequence using a dual-luciferase reporter assay (see 2.2.6.1) iii) Characterization of the molecular mechanism (mRNA slicing or translational arrest) responsible for the expression repression mediated by miRNA-133b.

3.5.2.1 miRNA-133b targets FAIM

FAIM is a widely expressed and evolutionarily conserved anti-apoptotic protein originally cloned from B-cells and with protective traits against Fas-mediated apoptosis (Schneider et al, 1999). *In-silico* analysis of the 3'UTR region of FAIM predicted the presence of an exclusive miRNA-133b binding site characterized by strong complementarity to the miRNA seed region (nucleotides 2-7). According to the prediction algorithm TargetScan this sequence in the 3´-UTR of FAIM displays a low degree of conservation among mammals (Figure 9A). In order to test if miRNA-133b and its predicted target molecule interact with each other, mRNA luciferase reporter constructs were generated. Cloning of the complete 3'UTR of FAIM into the psiCHECK-2 luciferase reporter plasmid rendered the luciferase (*Renilla reniformis)* after co-transfection of miRNA-133b inactive by 40%. As demonstrated by competition experiments, the interaction between the binding-site in the 3'UTR and miRNA-133b was sequence specific, since co-transfection of αmiRNA-133 but not the control αmiRNA fully restored the activity of the *Renilla reniformis* luciferase (Figure 9B). Moreover, confirming the results obtained by microarray analysis, miRNA-133b transfection of HeLa cells translated into specific down-regulation of FAIM mRNA demonstrated by qPCR (Figure 9D). This led to 70% reduction of FAIM protein levels as evidenced by Western blot analysis (Figure 10C). Both effects were sequence specific and could be blocked by addition of a specific miRNA-133b inhibitor (Figure 9C and D).

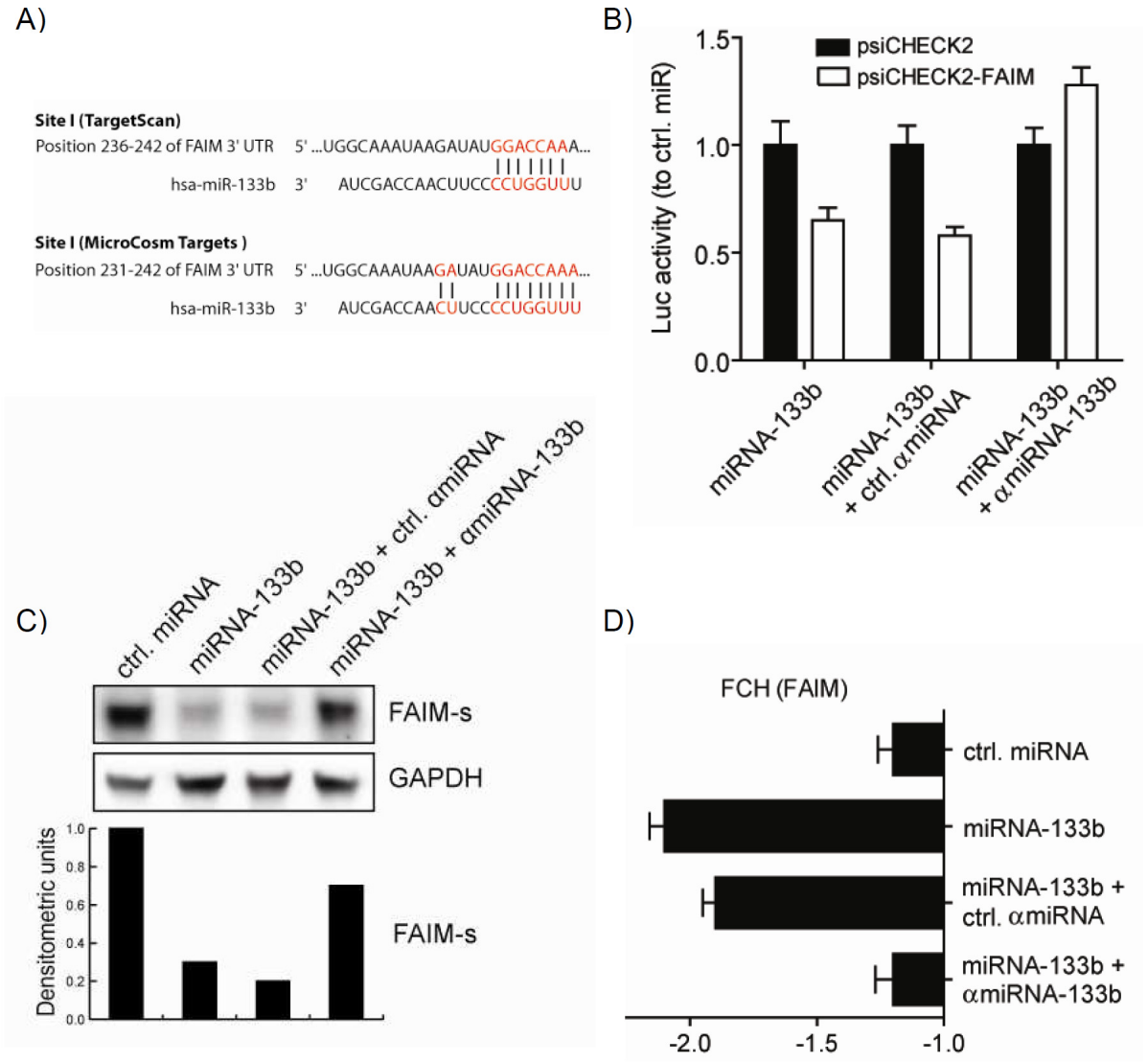


Figure 9. Expression of the anti-apoptotic molecule FAIM is regulated by miRNA-133b.

A) miRNA-133b target sites within the 3`UTR of FAIM as predicted by TargetScan and MicroCosm Targets. **B)** 3´-UTR targeting assay. HeLa cells were co-transfected with different combinations of miRNA mimics and a luciferase reporter plasmid harboring the complete 3´-UTR of FAIM (psiCHECK2-FAIM). The template plasmid (psiCHECK2) was used as a negative and normalization control. Activity of the 3´-UTR -dependent luciferase (*Renilla reniformis)* was measured 48 hrs. post-transfection and normalized for transfection efficiency to the one produced by the miRNA- and 3´-UTR-independent luciferase (*Photinus pyralis)*. Error bars = standard deviation **C)** Western blot and densitometric analysis of FAIM expression. Cells were transfected with miRNA-133b alone or together with a control antimiRNA (ctrl. αmiRNA) or a specific miRNA-133b inhibitor (αmiRNA-133b). After 48 hrs. cellular protein lysates were prepared and FAIM expression was assessed by Western blot. GAPDH was used as an internal loading standard. ctrl. miRNA transfected cells were used as a reference for densitometric quantification of protein band intensity. **D)** qPCR analysis of FAIM mRNA expression. Cells were transfected for 48 hrs. before total RNA was isolated, reverse transcribed and analyzed by qPCR for the expression of FAIM. Human acidic ribosomal protein (HuPO) was used as the house-keeping gene for internal normalization. qPCR values are shown relative to mock transfected cells incubated under the same conditions and were calculated by performing the ΔΔCt-method (Bookout et al, 2006). All graphs are representative of at least three independent experiments.

3.5.2.2 OPG expression is regulated by miRNA-133b

OPG is a soluble extracellular protein that acts as a decoy receptor (DcR) for TRAIL and protects cells from TRAIL-induced apoptosis (Emery et al, 1998). As revealed by microarray analysis, treatment of HeLa cells with miRNA-133b led to a -2.9 fold down-regulation of OPG transcripts (Table 10). This could be verified by qPCR analysis, where miRNA-133b treatment resulted in a specific reduction of up to three-fold of OPG mRNA levels 48 and 72 hrs. post-transfection (Figure 10A). In order to determine if the amount of protein was also impaired in a miRNA-133b dependent manner, enzyme linked immunosorbent assay (ELISA) was performed with cell culture supernatant of transfected cells. As a measure to avoid false positive results as a consequence of cytotoxicity caused by miRNAs or differential proliferation rates of transfected cells, MTT proliferation assay was used for normalizing the ELISA values to the amount of metabolically active cells per well. A reduction of 65 and 67% in the amount of secreted OPG at 48 and 72 hrs. after miRNA-133b transfection was observed, respectively. Co-treatment of the cells with the interfering αmiRNA-133b, but not the ctrl. αmiRNA, reconstituted the levels of released OPG almost completely back to those of ctrl. miRNA transfected cells (Figure 10B).

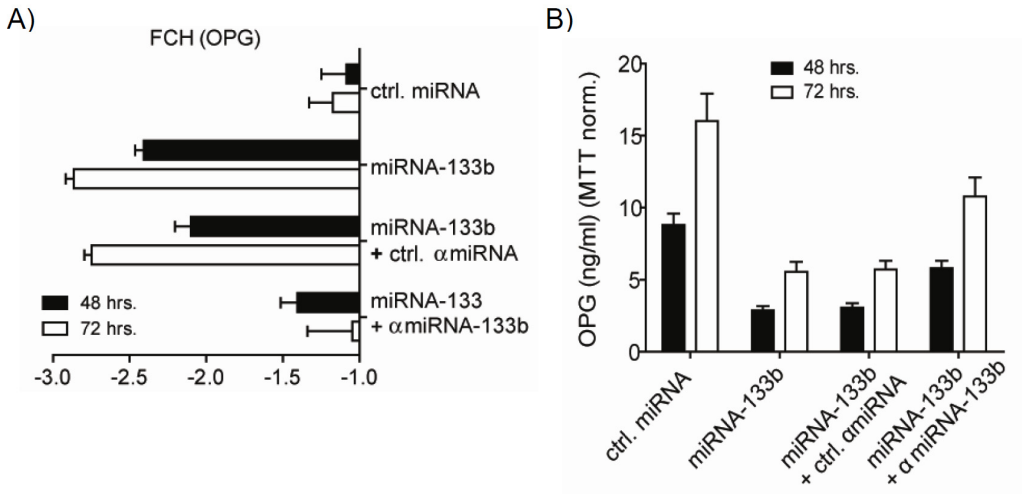


Figure 10. miRNA-133b is a post-transcriptional regulator of OPG.

Cells were transfected with miRNA-133b alone or together with a control antimiRNA (ctrl. αmiRNA) or a specific miRNA-133b inhibitor (αmiRNA-133b). Cells transfected with a scrambled control miRNA (ctrl. miRNA) served as point of comparison. **A)** At the indicated time points total RNA was isolated, reverse transcribed and analyzed by qPCR for the expression of OPG. Human acidic ribosomal protein (HuPO) was used as the house-keeping gene for internal normalization. qPCR values are relative to mock transfected cells incubated under the same conditions and were calculated by performing the ΔΔCt-method (Bookout et al, 2006). **B)** Cell culture supernatants of transfected cells were examined by ELISA for the presence of secreted OPG. At each time point, after collection of the supernatants a MTT assay was performed with the remaining cell layers. This was used for the normalization of the results obtained by ELISA. All graphs are representative of at least three independent experiments. Error bars = standard deviation

Unlike FAIM, analysis of the OPG 3´-UTR with several different target prediction databases did not reveal any potential miRNA-133 binding site. RNAhybrid is a tool for finding the minimum free energy hybridisation (mfe) of a long and a short RNA. The hybridisation is performed in a domain mode in which the short sequence is annealed to the best fitting part of the long one. The tool is primarily designed as a miRNA target prediction plattform (Rehmsmeier et al, 2004). Analysis of the 3'UTR of OPG with RNAhybrid pinpointed a single potential miR133b binding site (mfe = -18.8 kcal/mol) (Figure 11A; site I). Based on this, a luciferase reporter construct was generated and tested in co-transfection experiments with miRNA-133b. As measured by dual-luciferase assay, cloning of the complete OPG 3'UTR into the reporter construct did not impair the luciferase (*Renilla reniformis*) activity in miRNA-133b transfectants (Figure 11B). As an additional attempt to identify other binding sites, the search for a binding site was expanded to the remaining regions of the mRNA, specially the 5'UTR, as it has been recently proposed that it might also be involved in miRNA-mediated gene silencing (Lee et al, 2009a). An additional potential interaction motive displaying better structural features (mfe = -27.1 kcal/mol) was mapped. Besides having a lower mfe than the site localized in the 3´-UTR, this potential target sequence is localized exactly two nucleotides up-stream of the OPG start codon (Figure 11A; site II) which suggests that binding of miRNA-133b to this site might interfere with mRNA translation. In order to test this hypothesis special luciferase constructs were generated (see 2.2.6.2). Despite several attempts, manipulation of this region of the reporter plasmid always led to inactive luciferase transcription. Therefore, it was not possible to assess the importance of this sequence for miRNA-133b-mediated impairment of OPG expression

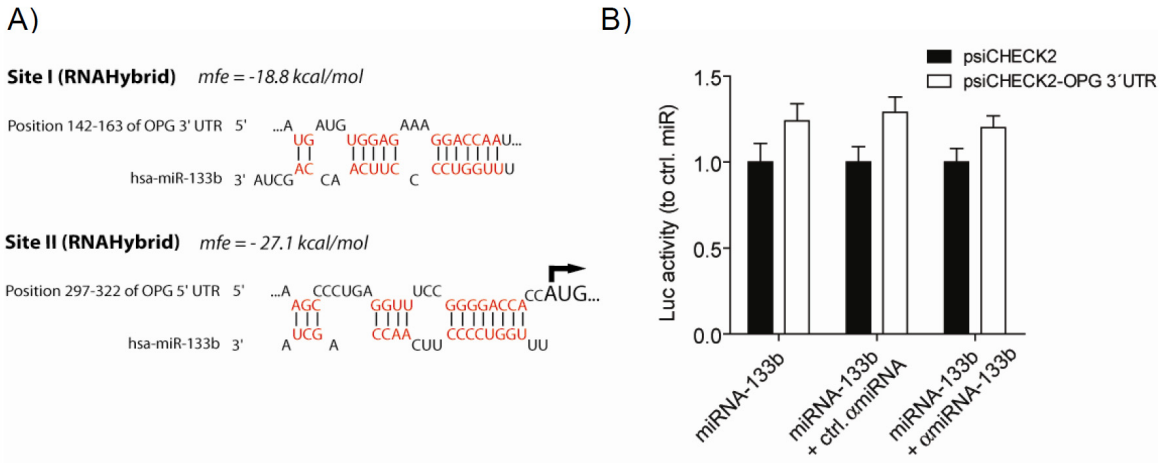


Figure 11. In-silico and in-vitro analysis of miRNA-133b binding sites in the OPG mRNA.

MiRNA-133b target sites within the 3´ and the 5´UTR of OPG as predicted by RNAhybrid **B)** 3´ and 5´UTR targeting assay. HeLa cells were co-transfected with different combinations of miRNA mimics and a luciferase reporter plasmid harboring the complete 3´-UTR of OPG (psiCHECK2-OPG). The template plasmid (psiCHECK2) was used as a negative and normalization control. Activity of the 3´-UTR -dependent luciferase (*Renilla reniformis)* was measured 48 hrs. post-transfection and normalized to the one produced by the miRNA- and 3´-UTR -independent luciferase (*Photinus pyralis)*. Results are representative of at least three independent experiments. Error bars = standard deviation. Arrow represente the translation inititation at the start codon.

Since OPG is an inhibitor of TRAIL-triggered apoptosis, it was possible that the hyperresponsiveness displayed by miRNA-133b transfected HeLa cells (Figure 5,6,7 and 8) was partly attributed to the diminished quantity of this decoy receptor in the supernatant. To verify this hypothesis, reconstitution experiments with recombinant human OPG (rhOPG) were performed with HeLa cells transfected with a ctrl. miRNA or miRNA-133b. 48 hrs. after transfection the cells were stimulated with rhTRAIL and either 100 or 1000 ng/ml rhOPG. As evidenced by PI-incorporation assays (Figure 12A) and measurement of the activation status of CASP8 and 3 (Figure 12B), addition of rhOPG to the culture medium was able to revert the exacerbated TRAIL-induced apoptotic response characteristic of miRNA-133b transfected cells. In case of the results provided by both readout systems the magnitude of the remaining apoptotic response corresponded exactly to that from control miRNA transfected TRAIL-stimulated vehicle-treated cells. This suggests that down-regulation of OPG by miRNA-133b is not the only mechanism contributing to the enhanced apoptotic response triggered by this DR ligand.

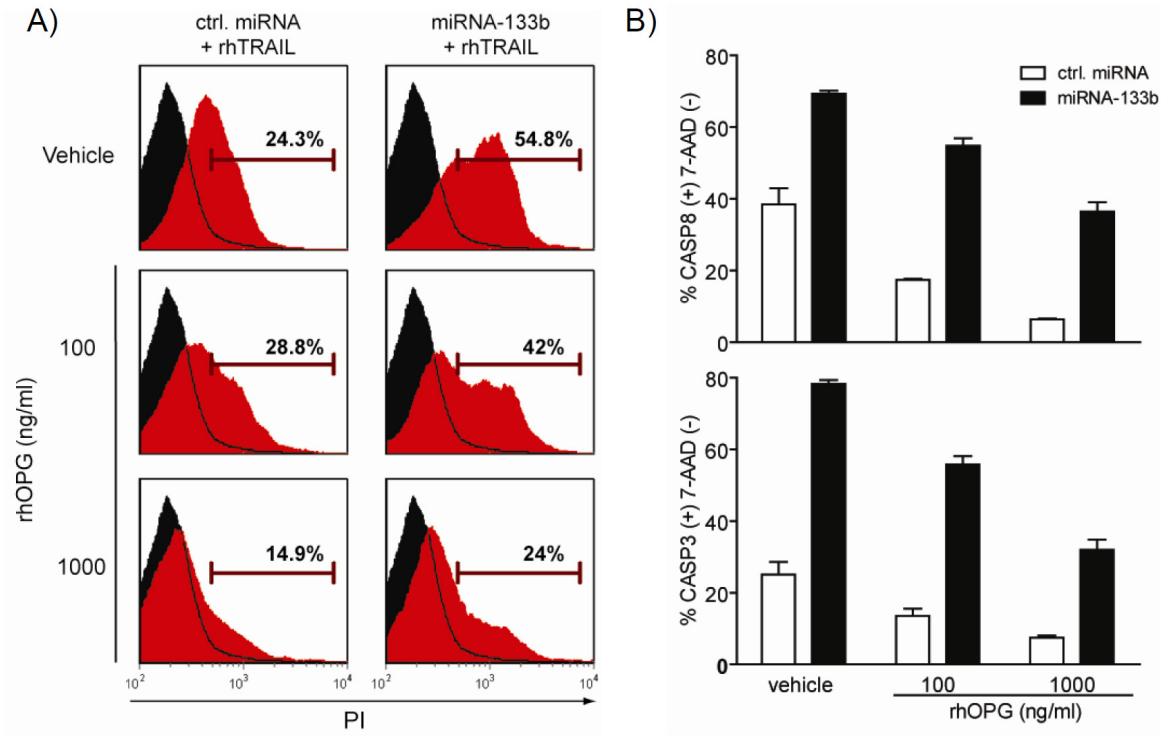


Figure 12. Recombinant OPG constrains the exacerbated TRAIL-responsiveness of miRNA-133b transfected cells.

Cells were transfected with a ctrl. miRNA or miRNA-133b mimic and incubated for 48 hrs. Transfected cells were stimulated with 20 ng/ml recombinant human TRAIL (rhTRAIL) both in the absence (vehicle: H_2O) or presence of 100 or 1000 ng/ml recombinant human OPG (rhOPG). **A)** Stimulated cells were collected, stained with propidium iodide (PI) and analyzed by flow cytometry. Samples were compared to unstimulated ctrl. miRNA transfected cells (black histogram). **B)** Treated cells were harvested, stained and scanned by flow cytometry for the presence of cleaved active CASP8 (upper graph) and 3 (lower graph). Cells transfected with ctrl. miRNA alone were used as a reference. Results are representative for at least three independent experiments. Errors bars = standard deviation.

3.5.2.3 FASN and GSTP1: two oncogenes regulated by miRNA-133b

FASN represents a very versatile enzyme fulfillling key tumorigenic functions (Menendez & Lupu, 2007). Microarray analysis of overall gene expression and mass spectrometric quantification of protein levels revealed reduced levels of FASN mRNA and protein in miRNA-133b transfected cells (Table 10 and 11). To validate these observations gene-specific qPCR and Western blot analysis of FASN were performed. Supporting the results obtained from the high-throughput approaches, treatment of HeLa cells with miRNA-133b reduced the levels of FASN mRNA in a sequence specific manner. This translated to 60% reduction at the protein level for single miRNA-133b transfected cells and, 20% when co-transfected with an unspecific αmiRNA. Introduction of the specific inhibitor of miRNA-133b instead, rescued and enhanced the production of FASN protein (Figure 13A and B).

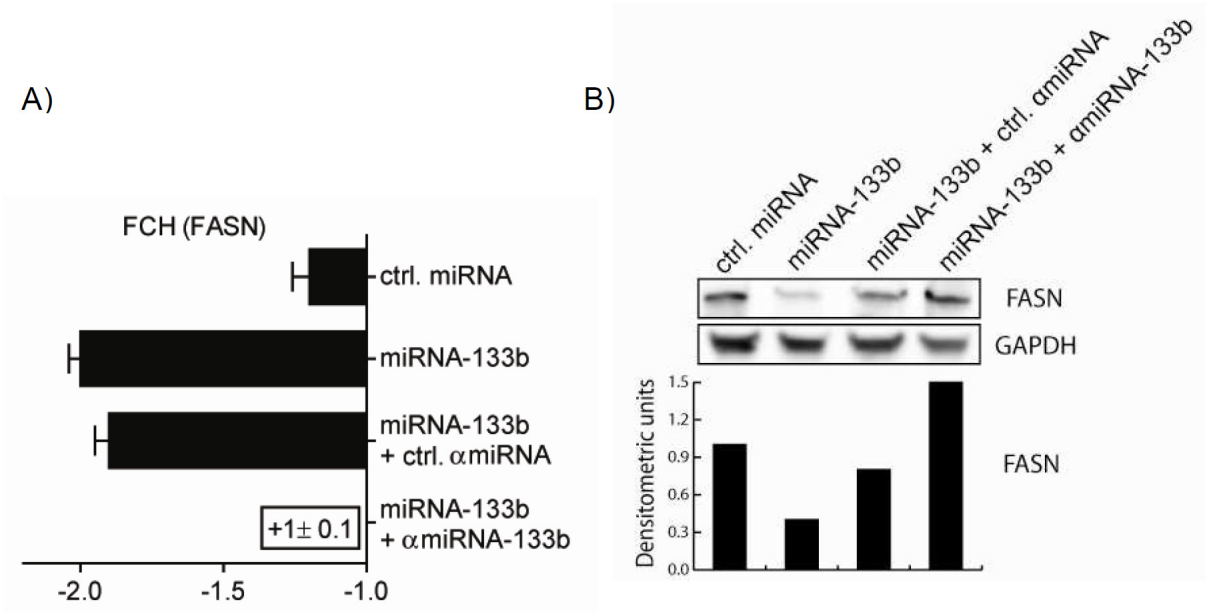


Figure 13. FASN expression is impaired by miRNA-133b.

Cells were transfected with miRNA-133b alone or together with a control antimiRNA (ctrl. αmiRNA) or a specific miRNA-133b inhibitor (αmiRNA-133b). Cells transfected with a scrambled control miRNA (ctrl. miRNA) served as point of comparison. **A)** Total RNA was isolated, reverse transcribed and analyzed by qPCR for the expression of FASN 48 hrs. after introduction of the miRNA mimics in HeLa cells. Human acidic ribosomal protein (HuPO) was used as the house-keeping gene for internal normalization. qPCR values are relative to mock transfected cells incubated under the same conditions and were calculated by performing the ΔΔCt-method (Bookout et al, 2006). **B)** Western blot and densitometric analysis of FASN expression. Cell protein lysates were prepared 48 hrs. post-transfection and FASN expression was assessed by Western blot. GAPDH was used as an internal loading control. Cells transfected with ctrl. miRNA were used as a reference for densitometric quantification of protein band intensity. All graphs are representative of at least three independent experiments.

In an effort to better dissect the molecular interaction between miRNA-133b and FASN mRNA, a miRNA-133b binding-site sequence analysis using RNAhybrid was performed. According to it, two potential binding regions with favorable biophysical characteristics (low mfe) were localized within the FASN mRNA. The first of them represented a canonical target site localized in the 3´-UTR of the transcript with an mfe of -28 kcal/mol (Figure 14A, site I). As assessed by luciferase assay in HeLa cells, cloning of the complete 3´-UTR of FASN into a reporter plasmid did not impair luciferase expression after co-transfection of miRNA-133b (Figure 14B). The second predicted binding site displayed an almost perfect complementarity to miRNA-133b (mfe = -33.6 kcal/mol) and was located within exon 33 of FASN cds (Figure 14, site II). Due to lack of an appropriate experimental system for validating miRNA targeting of sequences located within the cds of genes, a first attempt to prove the potential binding capacity of miRNA-133b to this site was assessed by introducing it as artificial 3´-UTR into psiCHECK2. According to luciferase reporter assay, co-transfection of HeLa cells with this construct and miRNA-133b did not affect the

production of *Renilla* luciferase, when compared the unmodified plasmid (Figure 14B). Hence the exact molecular mechanism by which miRNA-133b down-regulates the expression level of FASN could not be fully elucidated.

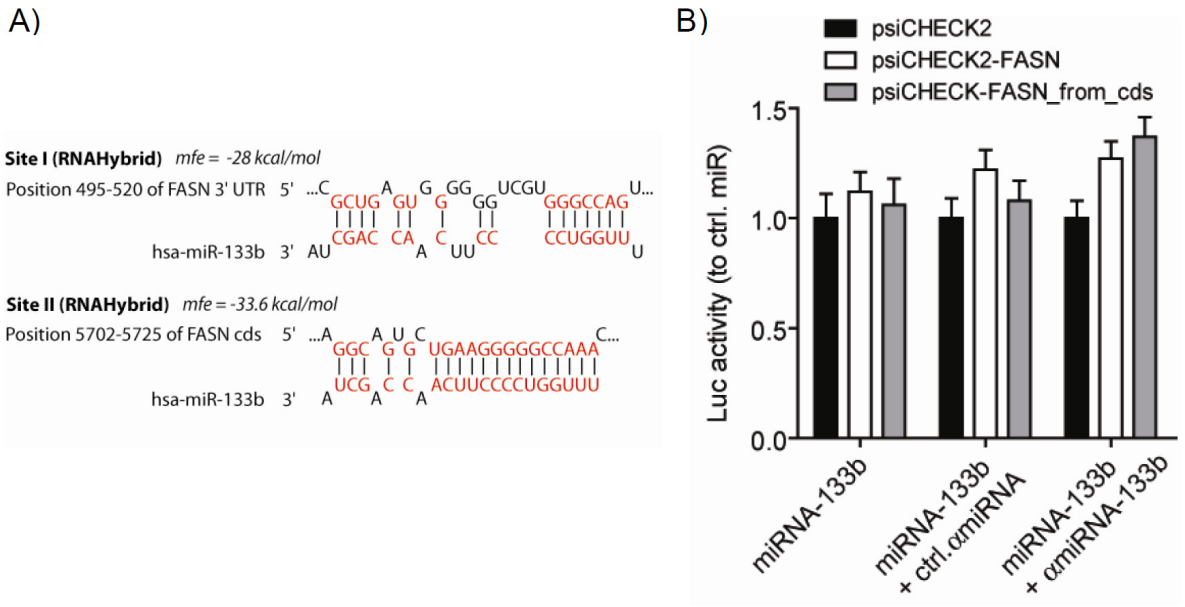


Figure 14. Sequence analysis of miRNA-133b binding sites within the FASN mRNA.

A) Predicted miRNA-133b target sites within the 3`UTR and the coding sequence (cds) of FASN as calculated by RNAhybrid **B)** miRNA-133b targeting assay. HeLa cells were co-transfected with different combinations of miRNA mimics and luciferase reporter plasmids harboring either the complete 3´-UTR of FASN (psiCHECK2-FASN) or the predicted target region in the cds (psiCHECK2-FASN_from_cds). The template plasmid (psiCHECK2) was used as a negative and normalization control. Activity of the 3´-UTR -dependent luciferase (*Renilla reniformis)* was measured 48 hrs. post-transfection and normalized to the one produced by the miRNA- and 3´-UTR -independent luciferase (*Photinus pyralis)*. Results are representative of at least three independent experiments. Error bars = standard deviation.

GSTP1 is an ubiquitously expressed protein that plays an important role in detoxification and xenobiotics metabolism (Wu et al, 2006). As predicted by TargetScan and MicroCosm Targets, GSTP1 has one poorly conserved miRNA-133b binding site in its 3´-UTR (Figure 15A). To confirm this, a 3´-UTR targeting luciferase reporter plasmid was constructed. Co-transfection of HeLa cells with the plasmid and miRNA-133b showed ~40% reduction of luciferase (*Renilla reniformis)* activity. Supporting a direct interaction between miRNA-133b and the cloned target sequence, co-treatment with a specific αmiRNA, but not a negative control, restored full enzymatic activity (Figure 15B). To further test the regulatory role of the molecular interaction between miRNA-133b and the 3´-UTR of GSTP1, mRNA and protein levels were determined after miRNA transfection. qPCR analysis confirmed a miRNA-133b dependent 2.3 fold reduction of the mRNA coding for GSTP1 (Figure 15D).

Accordingly, transfected cells showed 50% less GSTP1 protein (Figure 15C). Both observations matched the results obtained by microarray and pSILAC analysis (Table 10 and 11) providing a further line of evidence for the regulatory role of miRNA-133b over GSTP1 expression. Hence, miRNA-133b is a pro-apoptotic molecule with the ability to impair the expression of important oncogenes.

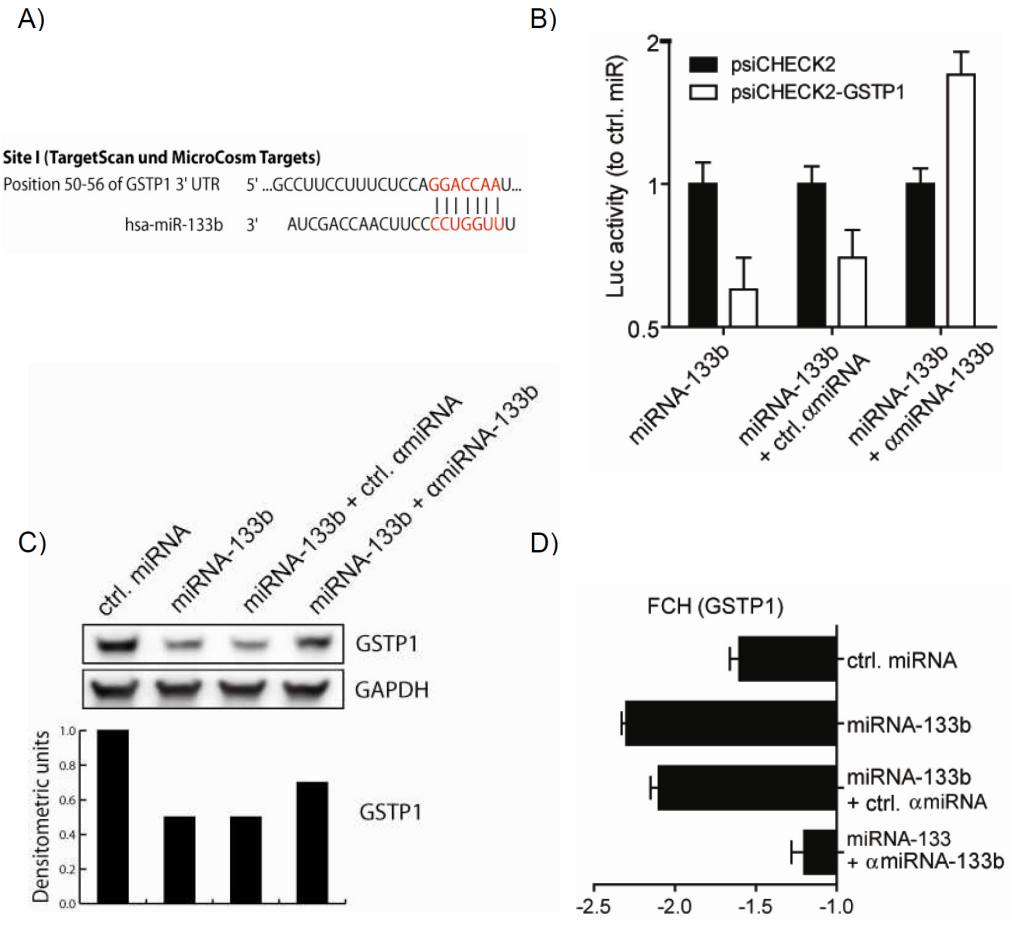


Figure 15. GSTP-1 expression is miRNA-133b dependent.

A) miRNA-133b target site within the 3´-UTR of GSTP1 as predicted by TargetScan and MicroCosm Targets. **B)** 3´-UTR targeting assay. HeLa cells were co-transfected with different combinations of miRNA mimics and a luciferase reporter plasmid harboring the complete 3´-UTR of GSTP1 (psiCHECK2-GSTP1). The template plasmid (psiCHECK2) was used as a negative and normalization control. Activity of the 3´-UTR -dependent luciferase *(Renilla reniformis)* was measured 48 hrs. post-transfection and normalized to the one produced by the control and 3´-UTR -independent luciferase (*Photinus pyralis)*. Error bars = standard deviation. **C)** Western blot and densitometric analysis of GSTP1 expression. Cells were transfected with miRNA-133b alone or together with a control antimiRNA (ctrl. αmiRNA) or a specific miRNA-133b inhibitor (αmiRNA-133b). 48 hrs. later cellular protein lysates were prepared and GSTP1 expression was assessed by Western blot. GAPDH was used as an internal loading control. Cells transfected with ctrl. miRNA were used as a reference for densitometric quantification of protein band intensity. **D)** qPCR analysis of GSTP1 mRNA expression. Cells were transfected for 48 hrs. before total RNA was isolated, reverse transcribed and analyzed by qPCR for the expression of GSTP1. Human acidic ribosomal protein (HuPO) was used as the house-keeping gene for internal normalization. qPCR values are relative to mock transfected cells incubated under the same conditions and were calculated by performing the ΔΔCt-method (Bookout et al, 2006). All graphs are representative of at least three independent experiments.

3.6 Activation of the innate immune response induces miRNA-133b expression

After having characterized the pro-apoptotic response mediated by miRNA-133b and having pinpointed several of the molecular mechanisms and interactions that account for it, the next aim was to gain a deeper insight into the regulation of miRNA-133b expression. The levels of several miRNAs have been previously demonstrated to depend strongly on activation of the innate immune system e.g. through stimulation of Toll-like receptors (TLRs) (O'Connell et al, 2007; Sheedy & O'Neill, 2008; Tili et al, 2007). To investigate whether miRNA-133b expression can be influenced by triggering the innate immune response, human THP1 macrophages were stimulated with various different TLR ligands. After stimulation total RNA was extracted from the cells and the expression level of miRNA-133b was analyzed by gene-specific qPCR. As a point of comparison the amount of miRNA-146a, a molecule well known to be strongly up-regulated during the pro-inflammatory response, was determined.

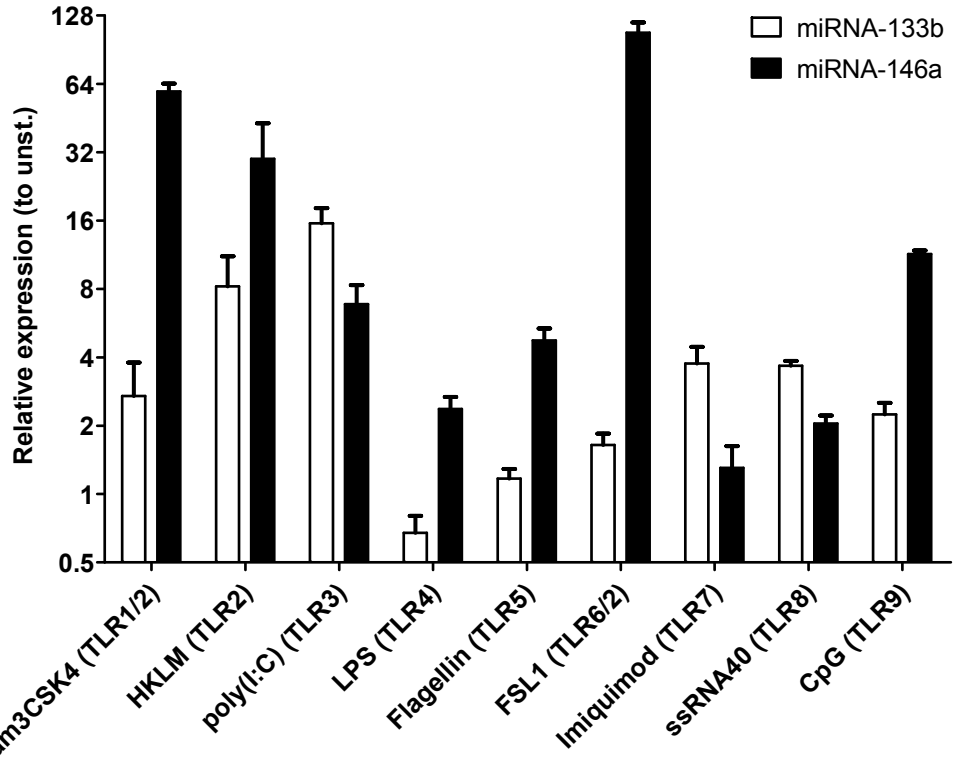


Figure 16. Expression levels of miRNA-133b and -146a after TLR stimulation.

THP1 macrophages were stimulated with 100 ng/ml Pam3CSK4, 10^8 cells/ml heat-killed *Listeria monocytogenes* (HKLM), 10 µg/ml poly(I:C), 100 ng/ml lipopolysaccharide (LPS), 1 µg/ml *S. typhimurium* flagellin, 100 ng/ml FSLI, 1 µg/ml imiquimod, 1 µg/ml ssRNA40 or 10 µg/ml CpG ODN2006. After 6 hrs. of stimulation cells were collected and total RNA was extracted. miRNA-specific qPCR was performed with RNU6B as an internal normalization control. Values represent the miRNA expression levels compared to unstimulated cells and were calculated by performing the ΔΔCt-method (Bookout et al, 2006).

In accordance to the results obtained by several other groups, activation of almost all TLRs led to increased levels of miRNA-146a. Treatment of cells with some specific stimuli also resulted in enhanced miRNA-133b expression. Activation with HKLM led to 8 fold increase, whereas eliciting of TLR1/2, 7, 6 and 9 with Pam3CSK4, imiquimod, ssRNA or CpG, respectively, showed less pronounced effects that ranged between twofold to fourfold induction (Figure 16). Notably, priming of the macrophages with poly(I:C), the cognate ligand of TLR3, provoked a 15 fold enhancement of miRNA-133b production. To test whether this was also the case in the cell system used so far, HeLa cells were treated with poly(I:C) and subsequently analyzed for the abundance levels of both miRNA-133b and -146a. Triggering of TLR3 in these cells, known to express all TLRs (Nishimura & Naito, 2005), led to 15 and 7 times more miRNA-133b and -146a respectively (Figure 17). In summary, miRNA-133b expression depended on activation of the innate immune system.

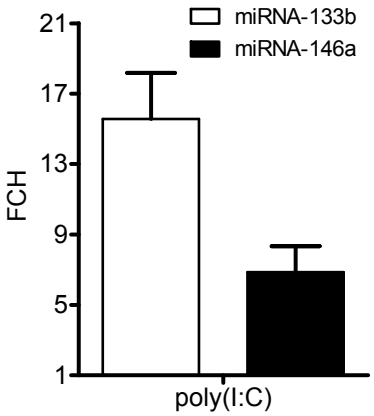


Figure 17. TLR3 activation of HeLa cells enhances miRNA-133b and -146a expression.

HeLa cells were stimulated. with 10 µg/ml poly(I:C) for 6 hrs. Primed cells were collected and total RNA was extracted. miRNA-specific qPCR was performed with RNU6B as an internal normalization control. Values represent the miRNA expression levels compared to unstimulated cells and were calculated by performing the ΔΔCt-method (Bookout et al, 2006).

3.7 miRNA-133b leads to enhanced NF-κB activity

Currently, the best characterized function of miRNAs whose expression is enhanced by TLR ligation is the down-regulation of key molecules of the signaling cascades that mount the innate immune response. By doing this, miRNAs provide a negative feedback mechanism that protects both the cell and organism from harmful exacerbated pro-inflammatory responses (Hou et al, 2009; Liu et al, 2009a; Taganov et al, 2006). NF-κB activation constitutes the most prominent step after TLR activation leading to establishment of an

inflammatory response. To test whether miRNA-133b can influence the activation level of NF-κB, luciferase activity assays were performed. To this end, HeLa cells were co-transfected with a reporter plasmid expressing the *Photinus pyralis* luciferase in an NF-κB dependent manner and different combinations of miRNA mimics (Yang et al, 1998). Two days after treatment the samples were either left untreated or stimulated with TNFα or poly(I:C) in order to assess the activation status of NF-κB. Introduction of miRNA-133b into HeLa cells led to enhanced NF-κB activity after TNFα or poly(I:C) treatment. MiRNA-133b transfectants were characterized by an average increase in NF-κB activity of about three and a half times after TNFα-stimulation and seven times after poly(I:C) treatment. This enhancement was particular for miRNA-133b and could be inhibited by addition of a sequence specific inhibitory αmiRNA, but not a scrambled one (Figure 18A). As an attempt to indirectly determine the activation stage of NF-κB, expression of two major pro-inflammatory targets was determined by qPCR. Supporting the results obtained with the luciferase assay, cells transfected with the miRNA-133b expressed four and seven times more interleukin 6 (IL6) and 8 (IL8) after TNFα-stimulation, respectively (Figure 18B).

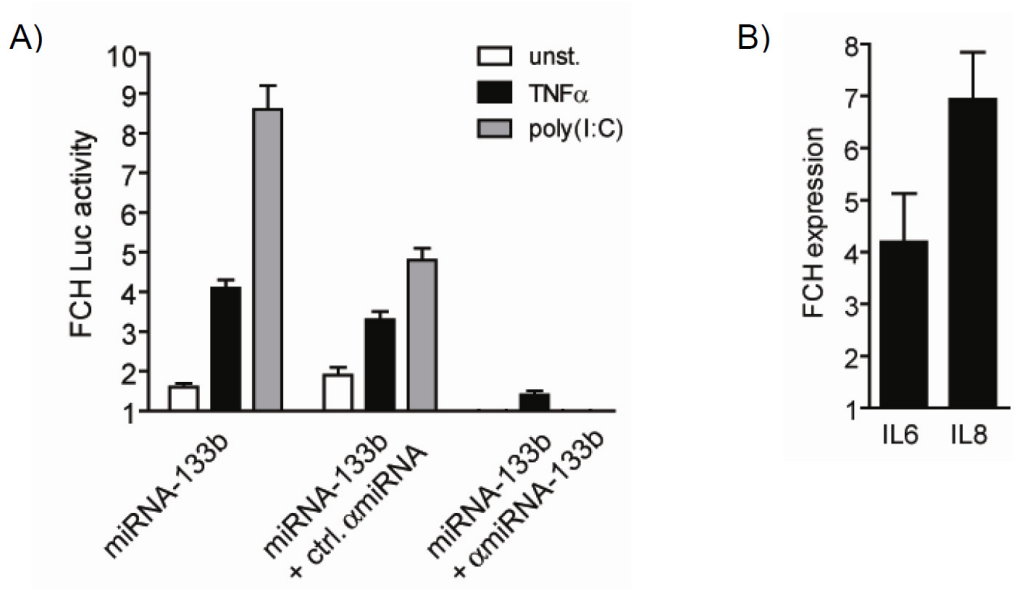


Figure 18. miRNA-133b enhances NF-κB activation and pro-inflammatory cytokine synthesis.

A) HeLa cells were co-transfected with different combinations of miRNA mimics and a NF-κB dependent luciferase (*Photinus pyralis)* reporter plasmid. A plasmid expressing the *Renilla reniformis* luciferase was also introduced as transfection efficiency normalizing control. Transfected cells were incubated for further 48 hrs. before they were either left untreated or primed for 4 hrs. with 20 ng/ml TNFα or 10 µg/ml poly(I:C). Cells transfected with ctrl. miRNA were used as a reference point for each different type of treatment. For assessing the activation status of NF-κB, all samples were lysed and dual-luciferase assay was performed. Error bars = standard deviation. **B)** Cells were transfected with a control miRNA (ctrl. miRNA) or miRNA-133b and kept for 48 hrs. Following incubation, samples were treated with 20 ng/ml TNFα for 4 hrs. Primed cells were collected and total RNA was extracted. qPCR analysis of interleukin 6 and 8 (IL6/8) was performed using human acidic ribosomal protein (HuPO) as an internal normalization control. Values represent the gene expression levels compared to ctrl. miRNA transfected TNFα-treated cells and were calculated by performing the ΔΔCt-method (Bookout et al, 2006). All graphs are representative of at least three independent experiments.

Within the miRNA research field miRNA-146a constitutes the best characterized example of an immunoregulatory molecule that acts by down-modulating the expression of key signaling molecules. miRNA-146a serves as a negative regulator of NF-κB mediated inflammation by impairing the signal transduction by targeting of indispensable genes such as IRAK1/2 and TRAF6 (Hou et al, 2009; Perry et al, 2008; Taganov et al, 2006; Williams et al, 2008). To test, whether miRNA-146a was able to block the enhanced NF-κB activity mediated by miRNA-133b, NF-κB luciferase reporter assays of cells transfected with the miRNA-133b in combination with miRNA-146a were performed after TNFα- or poly(I:C)-stimulation. In both cases, co-transfection of miRNA-146a mimicked the effect of αmiRNA-133b by completely abolishing the enhanced NF-κB activity characteristic of miRNA-133b transfectants (Figure 19).

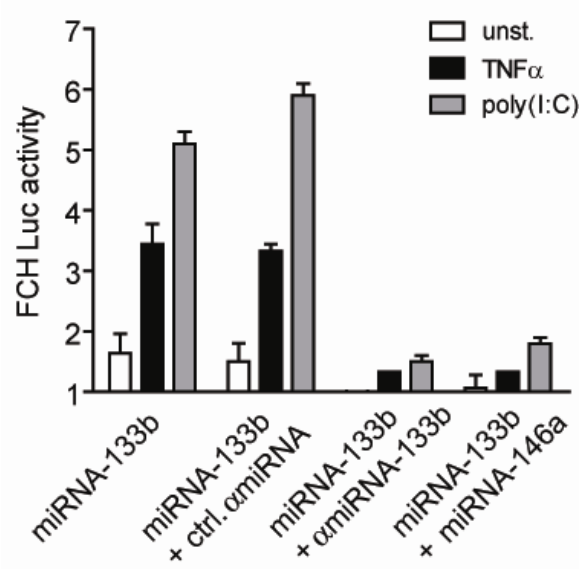


Figure 19. miRNA-146a blocks miRNA-133b mediated enhanced NF-κB activity.

A) HeLa cells were co-transfected with different combinations of miRNA mimics and a NF-κB dependent luciferase (*Photinus pyralis)* reporter plasmid. A plasmid expressing the *Renilla reniformis* luciferase was also introduced as transfection efficiency normalizing control. Transfected cells were incubated for further 48 hrs. before they were either left untreated or primed for 4 hrs. with 20 ng/ml TNFα or 10 µg/ml poly(I:C). Cells transfected with ctrl. miRNA were used as a reference point for each different type of treatment. For assessing the activation status of NF-κB, all samples were lysed and dual-luciferase assay was performed. Error bars = standard deviation. This result is representative for at least three independent experiments.

Summing up, miRNA-133b was able to improve the activation of NF-kB, leading to increased expression of pro-inflammatory cytokines. This implied that the expression of negative regulators of NF-κB might be impaired by miRNA-133b. Despite several attempts the identity of this molecule has remained elusive and further elaborated approaches are required.

4. Discussion

Ever since *lin-4,* the first identified miRNA, was discovered in 1993 (Lee et al, 1993; Wightman et al, 1993), this family of previously unknown molecules has earned a superordinate position among the central mechanisms of gene activity regulation. Since computational analyses predict that the expression of up to 30% of all genes is regulated by miRNAs, it is likely that they are involved in the control of all biological processes (Bartel & Chen, 2004). This work summarizes the results obtained by a series of experimental attempts aimed at identifying and characterizing miRNAs with immunomodulatory and apoptosis regulatory properties. This venture led to conclusive mechanistic evidence for miRNA-133b as a very versatile regulator of pro-apoptotic signaling events triggered by members of the TNFR superfamily and their ligands. The data presented herein constitute the first example of a single miRNA with the ability to influence all three major pathways of DR mediated apoptosis. In the following the relevance of these findings will be discussed within the context of current miRNA and immunological research.

4.1 miRNA-133b is a novel regulator of death receptor mediated apoptosis

Despite tremendous efforts to unravel the role of miRNAs as fine tuners of cellular fate and differentiation, to date only scant mechanistic evidence of their involvement in DR-triggered apoptosis exists. In the context of TNFα-induced apoptosis the only known example of miRNA involvement refers to the sensitization to TNFα cytotoxicity displayed by human embryonic kidney cells over-expressing the miRNA cluster miR-23a~27a~24-2 (Chhabra et al, 2009). Unfortunately, exact molecular mechanisms responsible for the increased apoptosis, *i.e.* target genes being down-regulated, still remain to be identified. With respect to the apoptotic response mediated by Fas it has been described that treatment of T-cells with an activating Fas-antibody induced expression of miRNA-143 in a time-dependent manner. Furthermore, over-expression of this miRNA in Jurkat cells translated to impaired proliferation and enhanced FasL-mediated apoptosis. In this case targeting of extracellular signal-regulated protein kinase 5 (ERK5) was proposed as the mechanism allowing a positive death-promoting feedback loop (Akao et al, 2009). The influence of miRNAs on DR-signaling has been mechanistically best characterized for the apoptosis inducing system composed of TRAIL and its receptors. At least ten different miRNAs have been identified as potential regulators (Corsten et al, 2007; Lu et al, 2009; Mott et al, 2007; Ovcharenko et al,

2007). Among these miRNA-221 und -222 were recently reported as over-expressed in more aggressive compared to less invasive tumors. It could be shown that these two miRNAs induce TRAIL resistance through targeting of phosphatase and tensin homolog (PTEN) and tissue inhibitor of metalloproteinases 3 (TIMP3) (Garofalo et al, 2009).

Herein, an *ex-vivo* model system of DR-triggered apoptosis was used to expand the knowledge on miRNA-based regulation of this key signaling pathway. By doing this miRNA-133b was characterized as a many-sided molecule with the ability to enhance cytotoxic responses triggered by TNFα, FasL or TRAIL. Encoded on the sixth human chromosome miRNA-133b and -206 compose a bicistronic miRNA cluster originally considered to be exclusively expressed in skeletal muscle (McCarthy, 2008). Recent reports support a broader expression pattern of both cluster members, and attribute miRNA-206 important regulatory functions in regions as different as brain, skeletal muscle or adipose tissue (Anderson et al, 2006; Hansen et al, 2007; Walden et al, 2009; Williams et al, 2009). Recently, miRNA-206 was brought into the context of apoptosis and cell cycle regulation by two independent publications demonstrating its capacity to promote apoptosis and to negatively regulate cell cycle progression (Adams et al, 2009; Song et al, 2009). In contrast to this profound mechanistic characterization of miRNA-206, miRNA-133b, the other cluster member, has remained by and large uninvestigated.

As demonstrated by our findings, the strong apoptotic response generated by transfection of miRNA-133b into HeLa cells is a direct consequence of multiple synergistic alterations of their protein repertoire *i.e*. miRNA-133b impairs the expression and function of various important anti-apoptotic genes. Here clear-cut evidence for FAIM as a target gene of miRNA-133b was provided since miRNA-133b transfection led to 70% reduction of FAIM protein levels in a sequence specific manner (Fig 9C). Originally discovered during a screen aiming to identify factors responsible for Fas-resistance in primary splenic B cells, FAIM has emerged as a highly conserved atypical regulator of DR-mediated apoptosis (Schneider et al, 1999). Presently almost no information is available about the mechanisms by which FAIM exerts its protective function. Its unique structure, with no significant primary sequence homology to any other known protein domains, does not allow any detailed mechanistic insight (Hemond et al, 2009). FAIM knockout mice are viable and have normal B- and T-cell populations. However, *faim*$^{-/-}$B cells and thymocytes show increased sensitivity to Fas-

triggered apoptosis *in-vitro*, and *faim*$^{-/-}$ mice suffer from greater mortality and exhibit exacerbated liver damage in response to Fas engagement *in-vivo*. Moreover, deletion of FAIM in mice results in greater activation of CASP8 and 3 and augmented PARP cleavage in FasL-stimulated, but not in dexamethasone, TNFα or γ-irradiated thymocytes (Huo et al, 2009). This resembles the phenotype observed in miRNA-133b transfected HeLa cells, where stimulation of the cells with an activating αCD95 antibody caused massive caspase activation and subsequent PARP degradation. At the same time, the observation made in the knockout mice implies that sensitization to TNFα detected in miRNA-133b transfected cells, as well as their enhanced responsiveness to TRAIL-stimulation, is probably not a direct consequence of FAIM down-regulation.

So far, two FAIM isoforms have been discovered. FAIM-L, the long variant, is present almost exclusively in neurons, whereas the short one (FAIM-S) displays a broader expression pattern (Segura et al, 2007; Zhong et al, 2001). Over-expression studies in neurons revealed a protective role of FAIM-L against TNFα and FasL mediated apoptosis upstream of both CASP8 and 3 and, the ability of FAIM-S to promote neurite outgrowth by a mechanism involving the activation NF-κB (Segura et al, 2007; Sole et al, 2004). Notably, both FAIM isoforms share the same 3'UTR containing one miRNA-133b binding site, implying that both protein variants could be subjected to post-transcritpional regulation by this miRNA. In fact, enriched presence of miRNA-133b was recently identified in the midbrain, where it regulates the maturation and function of dopaminergic neurons (Kim et al, 2007). Based on this and the miRNA-targeting evidence provided here, future work should investigate the importance of the down-modulation of FAIM by miRNA-133b during DR-triggered apoptosis and neuron development in the midbrain.

Two more functional attributes of FAIM have been characterized. First, *in-vivo* FAIM influences positively the expression of the key anti-apoptotic molecule cFLIP in lymphocytes and hepatocytes (Huo et al, 2009). cFLIP is known to antagonize Fas-signaling by interfering with recruitment of procaspase 8 (proCASP8) to the DISC (Scaffidi et al, 1999a). Reduced levels of cFLIP would allow a better physical interaction of proCASP8 with Fas thus leading to better caspase activation and probably enhanced apoptosis (Huo et al, 2009). To test, whether this was also the case in miRNA-133b transfected cells, cFLIP expression was analyzed (data not shown). No significant difference of cFLIP between ctrl. miRNA and

miRNA-133b transfected cells could be observed at the mRNA or protein level. A possible explanation for this could be that generation of a knockout mouse leads to complete absence of the deleted gene, whereas transient miRNA treatment merely results in a partial reduction (~70%) of protein expression (Figure 9). Therefore, miRNA-133b transfected cells might still contain sufficient FAIM to assure the expression of cFLIP. This would mean that induction of cFLIP expression is not the only mechanism by which FAIM protects cells from cytotoxic signals triggered by DR ligands. On the other hand, as observed in the FAIM knockout mouse, it is also feasible that up-regulation of cFLIP by FAIM is a specific anti-apoptotic mechanism of hepatocytes, thymocytes and B-lymphocytes. The second recently described FAIM function refers to its ability to enhance, both *ex-* and *in-vivo,* CD40 signaling for NF-κB activation, interferon regulatory factor 4 (IRF4) expression and B-cell lymphoma 6 (BCL6) down-regulation during B-cell differentiation processes (Kaku & Rothstein, 2009). Since miRNA-133b represents the first identified post-transcriptional regulator of this anti-apoptotic molecule and, considering the high degree of conservation across different species of both miRNA-133b and FAIM, it would be interesting to assess the relevance of miRNA-133b for processes associated to B-cell development.

Further evidence for the relevance of miRNA-133b as a controller of DR-mediated apoptosis was provided by the experiments presented here showing that it duplicates the apoptotic response induced by TRAIL in transiently transfected HeLa cells (Figures 5, 6, 7 und 8). OPG was originally described as a soluble decoy receptor with proficiency to inhibit osteoclast differentiation by interfering with the signaling system involving receptor activator of NF-κB (RANK) and its ligand (RANKL) (Leibbrandt & Penninger, 2008). Later on, with the discovery that OPG can also bind to TRAIL, it emerged as an important anti-apoptotic and tumor survival factor (Emery et al, 1998; Holen & Shipman, 2006). In this work it could be established that miRNA-133b impairs OPG transcription and expression. Hence, ~70% less decoy molecules are secreted to the medium by transfected cells (Figure 10). By adding the recombinant human OPG to the culture medium, it was possible to reconstitute the TRAIL resistance of miRNA-133b transfected cells. Importantly, despite addition of OPG significantly higher apoptosis levels were still observed in miRNA-133b transfectants, compared to equally stimulated ctrl. miRNA transfected cells (Figure 12). This suggests that impaired OPG expression is not the only mechanism contributing to exacerbated TRAIL responsiveness. This was further supported by the fact that specific down-regulation of OPG

using small-interfering RNAs (siRNAs) was not sufficient for mimicking the enhanced reactivity to TRAIL mediated by miRNA-133b (data not shown). Hence, the strong pro-apoptotic influence of miRNA-133b on TRAIL-mediated signaling is rather the consequence of pleiotropic down-modulation of gene activity, rather than being mediated by reduced activity of one single genetic entity.

Since OPG has a very strong affinity for RANKL, it is feasible that miRNA-133b mediated regulation of this decoy receptor might also be relevant in some of the numerous biological processes controlled by this cytokine. In line with this idea, it was recently shown that miRNA-206, the molecule belonging to the same cluster as miRNA-133b, performs important functions during osteoblast differentiation (Inose et al, 2009). Since OPG is a key player during the process of bone generation and resorption, and given that miRNA-133b should be co-expressed with miRNA-206, it is interesting to speculate on a possible function of the molecular regulatory system described in this work during skeletal remodeling.

Recently, further mechanistic evidence of a pro-apoptotic function of miRNA-133b was discovered. According to it, miRNA-133b is a potent death-promoting molecule that impairs the expression of the anti-apoptotic genes MCL1 and BCL-w. By doing this, miRNA-133b jeopardizes the preservation of mitochondrial membrane integrity during drug-induced apoptosis (Crawford et al, 2009). Importantly, this identification of miRNA-133b as a sentinel of the endogenic apoptotic pathway upholds the findings presented in this thesis. It strengthens the notion of miRNA-133b as death-promoting factor that fulfills its task by impairing the correct function of pro-survival responses on different levels from both endogenic and exogenic apoptotic pathways.

4.2 Tumor suppressor activity of miRNA-133b

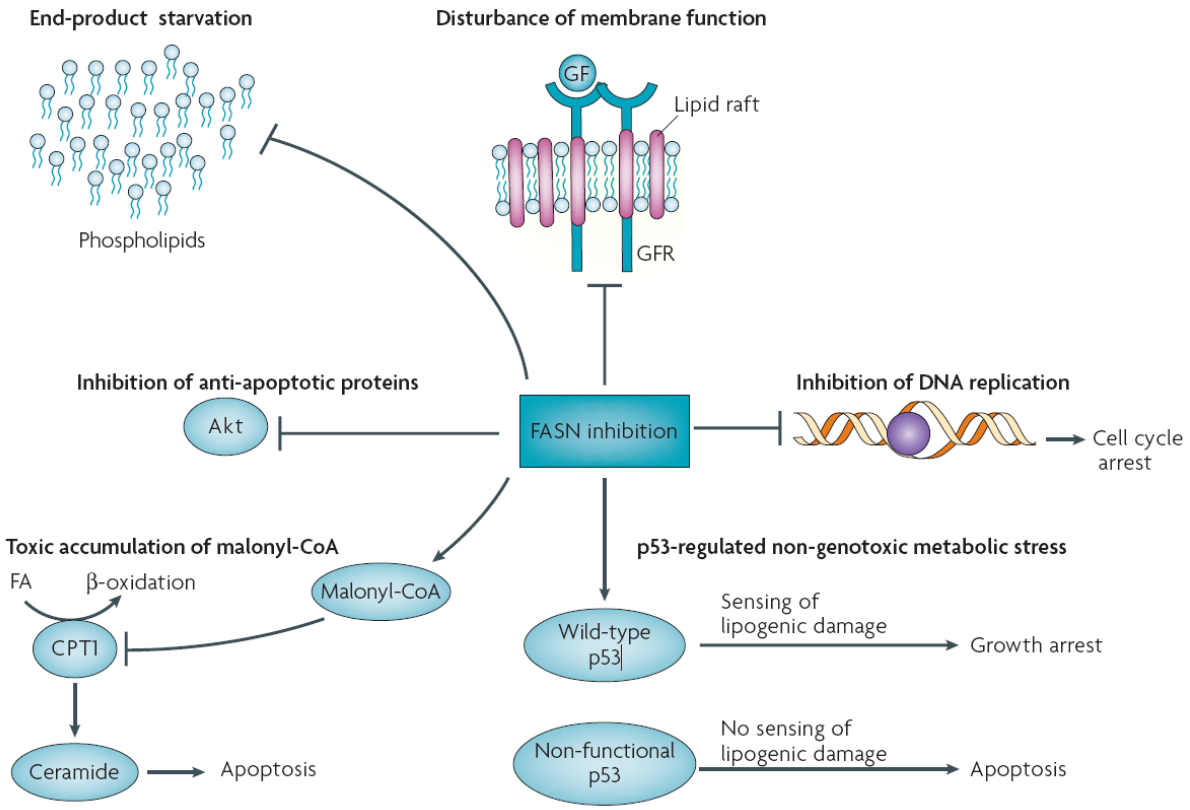
Cancer is a complex genetic disease caused by the accumulation of mutations, which lead to deregulation of gene expression and uncontrolled cell proliferation (Lee & Dutta, 2009). During the last years huge amounts of experimental evidence have accumulated that support the tight link between tumorigenesis and miRNA expression and function (Esquela-Kerscher & Slack, 2006; Lee & Dutta, 2009). The importance of miRNAs for cancer development is highlighted by the fact that approximately 50% of the genes encoding

miRNAs are located in cancer associated genomic regions or fragile sites, which are frequently amplified or deleted in tumors (Calin et al, 2004; Sevignani et al, 2007). According to their molecular function miRNAs can be classified as oncogenic or tumor-suppressing. One important example of a miRNA with tumorigenic activity is miRNA-21. It is up-regulated in almost all kinds of cancer where it inhibits apoptosis, promotes growth and induces a metastatic phenotype (Selcuklu et al, 2009). On the other hand, tumor-suppressing miRNAs like miRNA-15, -29 or -34b/c can show a reduced expression or even complete absence in neoplastic tissues. As common characteristic, most of them induce cell-growth arrest and enhance the apoptosis sensitivity of the tumors (Cimmino et al, 2005; Mott et al, 2007; Raver-Shapira et al, 2007).

Herein, it was demonstrated that miRNA-133b causes apoptosis and is therefore likely to have potent tumoricidal properties. According to this, one would expect that the expression levels of this miRNA should be reduced in cancerous tissue compared to healthy one. Indeed, all miRNA profiles of different cancer types published so far, in which miRNA-133b has been detected as differentially regulated, concur in a lower expression of this molecule in diseased tissue (see appendix 7.1). Some tumor types displaying this characteristic include ovary, breast, head and neck/oral, bladder, colorectal, testes and lung cancer (Bandres et al, 2006; Crawford et al, 2009; Kozaki et al, 2008; Navon et al, 2009; Tran et al, 2007; Wong et al, 2008a; Wong et al, 2008b). Thus, based on our data and considering the consistent down-regulation of miRNA-133b in different cancers affecting different types of organs, it is feasible to postulate the impairment of miRNA-133b expression as one of the multiple steps leading to tumor formation. In other words, reduced levels of miRNA-133b would favor tissue transformation by protecting cancerous cells from apoptosis. In the future it would be interesting to study this in more detail using mice models in which miRNA-133b locus has been genetically ablated.

In addition to FAIM and OPG other key genes whose expression is affected by miRNA-133b could be pinpointed in this work. The fact that transfection of miRNA-133b into HeLa cells resulted in 60 and 50% less FASN and GSTP1, respectively, stresses its importance as tumor suppressor (Figures 13 and 15). FASN is a central metabolic enzyme responsible for the final catalytic step during fatty acid synthesis. Compared with normal tissues, where FASN expression is very low, cancerous cells display much higher levels of it

(Kuhajda et al, 1994; Milgraum et al, 1997). In tumors, FASN plays important tumorigenic functions some of which include the generation of phospholipids needed for the cell membrane synthesis of fast growing cancer cells, formation of raft-aggregates implicated in key cellular and pro-survival signaling processes and prevention against accumulation of the tumoricidal sphingolipid ceramide (Bandyopadhyay et al, 2006; Pizer et al, 1996; Swinnen et al, 2003) (Scheme 13). From this perspective, miRNA-133b mediated suppression of FASN expression is very important, since it impairs the proliferation capacity of cancer cells. Moreover, it renders tumors more prone to cell death both without and after DR stimulation. The former is relevant for the studies presented in this work, since it would explain why unstimulated miRNA-133b transfected cells displayed low but still significantly higher levels of apoptosis, compared to ctrl. miRNA transfected cells. This idea is supported by results obtained from chemical inhibition and gene knock-down experiments, which to some extent resemble miRNA-mediated gene silencing, where FASN inhibition resulted in CASP8 mediated tumor cell apoptosis (Knowles et al, 2008).



Scheme 13. FASN inhibition activates several mechanisms leading to tumor cell death (Menendez & Lupu, 2007)

GSTP1 belongs to a family of enzymes responsible for detoxification and protection of cells from harmful reactive species (Townsend & Tew, 2003). Of importance, GSTP1 expression is highly elevated in neoplastic tissue and has been implicated in resistance to apoptosis (Cumming et al, 2001). Recently, GSTP1 was reported to regulate TNFα triggered signaling through interaction with TNF receptor-associated factor 2 (TRAF2) both *ex- and in-vivo.* As a consequence of this, activation of apoptosis signal-regulating kinase 1 (ASK1) is impaired and, an essential axis for TNFα mediated apoptosis is blocked (Wu et al, 2006). Hence, by modulating the expression of two important oncogenes like FASN and GSTP1, miRNA-133b jeopardizes the ability of highly proliferating tumors to manage chemical stress and to self-supply with essential biomolecules. This feature together with the ability to control actively the sensitivity of cells to DR-stimulation may explain why down-regulation of miRNA-133b is a common feature in several different tumor types.

4.3 miRNA-133b involvement during the innate immune response

In the context of innate immunity accumulating evidence has revealed pivotal roles of miRNAs during the establishment, maintenance, regulation and termination of primary defense responses against invading pathogens (O'Connell et al, 2010; Sonkoly & Pivarcsi, 2009). Some prominent examples of miRNAs involved in the modulation of innate immunity include let-7e, miRNA-9, -34, -146 and -155 among others. These molecules have been shown to be involved in processes such as regulation of TLR-expression, NF-κB activation, DC differentiation, inflammation and binding ability to pathogens (Androulidaki et al, 2009; Bazzoni et al, 2009; Hashimi et al, 2009; Martinez-Nunez et al, 2009; Taganov et al, 2006). Hence, miRNAs have become a very powerful tool for studying and understanding how the fine tuning of the innate immune system takes place and constitute interesting targets for the development of novel therapies against immunological disorders.

Hitherto, no connection of miRNA-133b with the innate immune system has been drawn. By stimulating monocytic-like cells with TLR agonists it was shown that the amount of miRNA-133b in the cell can be strongly influenced by activation of the cells with pathogen associated molecular patterns (PAMPs) (Figure 16). Moreover, *in-vitro* infection of macrophages with non-pathogenic *M. bovis* BCG, but not pathogenic *M. tuberculosis,* also induced strong miRNA-133b expression during the first 24 hrs. of infection (This finding will

be discussed in the next section) (Table 9 and Figure 1). This correlation between activation of the innate immune system and miRNA-133b up-regulation strongly implies the latter might play an immunoregulatory role during the inflammatory response triggered after TLR stimulation. Currently, most of the miRNAs described in the context of inflammation and innate immunity have been characterized as negative regulators of molecular signaling cascades leading to production of pro-inflammatory cytokines like IL1β, IL6 or TNFα. By doing so, miRNA-146a or -147 protect cells from excessive counterproductive innate immune responses because their own expression is up-regulated after activation of the inflammatory response (Liu et al, 2009a; Taganov et al, 2006). Contrary to this, the data presented here showed miRNA-133b as a small molecule with the ability to positively regulate and enhance the NF-κB signaling triggered by TNFα or poly(I:C). As a consequence of this, four and seven times more IL6 or 8 were synthesized, respectively (Figure 18B). Thus, miRNA-133b represents a novel class of immunorelevant miRNA whose role is not to block the activation process triggered after pathogen recognition, but rather to boost it.

As demonstrated by the NF-κB luciferase assays, miRNA-146a is able to block the hyper activation observed as a consequence of increasing the intracellular levels of miRNA-133b (Figure 19). This raises the question about the interaction between co-expressed miRNAs and the biological consequences of this. One possibility, as shown here, is that co-existing molecules antagonize each other as a mechanism to avoid a dangerous polarization of the cellular response. In this case, the final outcome of the response would strongly depend on the kinetics of induction of the miRNAs and their target molecules. Furthermore, biochemical and biophysical properties such as stability and half-life of the molecules would also benefit the one or other direction of the cellular response. Thus, the biological effect of miRNA-133b and its pro-inflammatory activity might not only depend on whether it is expressed or not, but also on the co-existence of other miRNAs.

Herein it was shown that the NF-κB hyperactivity was specific for miRNA-133b transfectants (Figure 18A). Based on this, it is tempting to postulate a negative regulator of NF-κB as one of the genes targeted by miRNA-133b. Indeed, many interesting NF-κB regulators such as tumor necrosis factor inducible protein A20 (A20) or protein phosphatase 2 catalytic subunit alpha isoform (PP2A) show a good complementarity to miRNA-133b and are predicted as potential targets *in-silico* (Barisic et al, 2008; Song et al, 1996). In order to

test this possibility, 3´-UTR luciferase reporter constructs were generated and the mRNA expression and protein levels of several of these candidate genes was analyzed after transfection of HeLa cells with miRNA-133b (data not shown). Unfortunately, despite several attempts none of the predicted targets showed a miRNA-133b-dependent expression pattern. Hence, the identity of the negative regulator of NF-κB being down-regulated after miRNA-133b treatment has remained elusive. Its identification will be of crucial importance for the understanding of phenotype caused by miRNA-133b.

Further hints of a potential involvement and importance of miRNA-133b as a regulator of innate immune mechanisms are provided by the molecules whose expression is impaired by it. As mentioned previously, OPG has a high affinity for RANKL and this interaction is of crucial importance for bone homeostasis. Besides its pivotal role as central regulator of osteoclast development and function, RANKL is also of extreme importance in various processes associated to innate immunity. It augments the ability of DCs to stimulate naive T-cell proliferation, regulates the production of pro-inflammatory cytokines and potentiates the antigen presentation capacity of macrophages (Anderson et al, 1997; Maruyama et al, 2006; Park et al, 2005). Similarly, TRAIL, the other OPG ligand, also executes central functions associated with the innate immune system. For instance, TRAIL is up-regulated on monocytes and macrophages after stimulation with LPS or IFNβ (Ehrlich et al, 2003; Halaas et al, 2000). Furthermore, IFNγ stimulation induces TRAIL on the surface of monocytes, DCs and natural killer (NK) cells (Fanger et al, 1999; Griffith et al, 1999). Importantly, membrane bound TRAIL displayed by NK cells constitutes one of the main killing mechanism used by these cells to combat tumors (Smyth et al, 2001). Future work should explore the importance of miRNA-133b in the aforementioned processes. An important task here will be to determine the expression levels of miRNA-133b among the different cell types and under distinct activation stages of the immune system.

4.4 miRNA-profile of mycobacterial infection

Despite tremendous accumulating evidence of the importance of miRNAs for the appropriate function of the immune system, currently no data are available on the role they could play during mycobacterial infections. By performing miRNA-expression analysis of macrophages infected either with pathogenic *M. tuberculosis H37Rv* or apathogenic *M. bovis*

BCG, several miRNAs displaying an altered expression profile after 24 and 48 hrs. infection were identified (Table 9 and Figure 1). The list of miRNAs differentially expressed included molecules that have been previously well characterized in the context of innate immunity *e.g.* miRNA-146a, -155 and let-7e (Androulidaki et al, 2009; Martinez-Nunez et al, 2009; O'Connell et al, 2007; Taganov et al, 2006). Notably, also species with no previous record of immunoregulatory activity could be detected. Most differentially regulated miRNAs showed subtle expression changes compared to uninfected cells. Rather than being an intrinsic characteristic of the experimental system used here, this has become a general observation of different groups performing miRNA profiling experiments. The current consensual model in the miRNA field is that even marginal expression level changes may lead to severe effects and phenotypes (Baek et al, 2008; Linsen et al, 2008; Selbach et al, 2008). The reason for this has been attributed to the pleiotropic nature of miRNA regulatory function which allows these short RNAs to interfere, or promote, simultaneously different molecular mechanisms and signaling pathways. Hence, even small changes like those observed for most miRNAs detected with our setup of mycobacterial infection might be relevant.

Currently, the study of miRNA function and relevance during *M. tuberculosis* infection is particularly hindered by the difficulty to perform transfection and over-expression studies in biologically relevant host cells *i.e*. macrophages and DCs (Lee et al, 2009b; Zhang et al, 2009). As an alternative to circumvent this technical issue it was decided to first screen candidate miRNAs for regulatory properties in the context of TNFα mediated apoptosis and signaling using the easily transfectable and well characterized HeLa cell system. TNFα was chosen since it plays pivotal roles in the initial and long-term control of TB. For instance, gene knockout mice deficient in TNFRI suffer from exacerbated TB (Balcewicz-Sablinska et al, 1998; Flynn et al, 1995a; Kaufmann, 2002; Lin et al, 2007). Using this experimental setup miRNA-133b was identified as a strong sensitizing agent against TNFα induced cell death (Figures 2, 3 and 4). Based on this observation the development of a lentiviral transduction platform for over-expressing miRNA-133b in human macrophages was started. Unfortunately, generation of these constructs proved to be very challenging, mainly because of cloning difficulties related to the complex irregular hairpin structure of miRNA precursors that is essential for correct processing and generation of biologically active molecules. Hence generation of stable miRNA-133b over-expressing macrophages is still underway. These cells will allow the characterization of the involvement of this potent pro-apoptotic molecule during the innate immune response to *M. tuberculosis*.

As shown by our results, miRNA-133b was strongly induced by macrophages after 24 hrs. infection with the vaccine strain *M. bovis* BCG, but not virulent *M. tuberculosis* H37Rv. The importance of macrophage apoptosis for the outcome of mycobacterial infection is still unclear because ambivalent experimental evidence has been collected in the past. *M. tuberculosis* could act as pro-apoptotic pathogen that induces cell death in order to escape from macrophages and promote extracellular spread of the infection. Indeed, primary human alveolar macrophages obtained from bronchoalveolar lavage of TB patients show elevated apoptosis levels when compared to healthy control individuals (Klingler et al, 1997; Placido et al, 1997).

Moreover, cellular death induction in infected macrophages seems to rely on a novel caspase-independent pathway that shares features of both apoptosis and necrosis (Lee et al, 2006; O'Sullivan et al, 2007). Thus, there is evidence available that supports the concept of *M. tuberculosis* as an apoptosis inducing pathogen. Remarkably, the magnitude of the cell death inducing response seems to be strongly dependent on the genetic background of the host cell, the bacterial strain and the multiplicity of infection used (Briken & Miller, 2008). In contrast to this, prevention of host apoptosis could represent a strategic tool of virulent mycobacteria to evade a key-step of innate immunity and perpetuate their replication niche. Several lines of robust evidence support the blockade of host cell apoptosis as an important virulence mechanism of *M. tuberculosis*. For instance, it is known that pathogenic mycobacteria can induce anti-apoptotic genes much stronger than non-virulent high apoptosis inducing strains (Dhiman et al, 2007; Sly et al, 2003; Spira et al, 2003). Furthermore, *M. tuberculosis* inhibits the extrinsic apoptotic pathway by modifying the expression of death receptors such as Fas and soluble TNF receptor 2 (sTNFR2) (Balcewicz-Sablinska et al, 1998; Oddo et al, 1998).

Finally, the strongest evidence for the anti-apoptotic nature of *M. tuberculosis* was provided by the identification of three genes (*nuoG, soda and PknE)* in its genome that encode proteins responsible for reduced levels of infection-triggered apoptosis (Hinchey et al, 2007; Jayakumar et al, 2008; Velmurugan et al, 2007). In summary, several lines of conclusive evidence support the ability of mycobacteria to actively manipulate the apoptotic response of its host. This seems to occur according to the necessity of intracellular mycobacteria to either replicate safely within the macrophage or, abandon it and disseminate. Given their pivotal role for the regulation of the apoptotic program, miRNAs, including

miRNA-133b, could be used for a better dissection of the molecular mechanisms that allow mycobacteria to control the fate of the host cell. Moreover, active manipulation of the miRNA repertoire of the target cell by the invading pathogen could represent a previously unforeseen mechanism of host manipulation. In fact, this has already been described for the bacterial plant pathogen *Pseudomonas syringae.* This pathogen has developed the ability to inject effector proteins into the host cell that allow it to manipulate the host miRNA machinery (Navarro et al, 2008). In order to approach this question, the development of suitable miRNA over-expression and repression tools for use in human macrophages and DCs is imperative.

Besides the potential of miRNAs as tools for deciphering and approaching host-pathogen interactions, they could also provide valuable information about the immune status of the host cell and even the whole organism. From this perspective it is feasible to envision miRNA signatures of TB patients as a possible way of characterizing the disease state. Moreover, such expression profiles could have both diagnostic and prognostic value. Indeed, first studies have already determined that miRNAs can be even more accurate than traditional expression profiles when classifying tumors according to their developmental lineage (Lu et al, 2005). Even more, the level of certain miRNAs can help predict the effectiveness of pharmacological treatment. This has been demonstrated in human biopsies of hepatitis C patients, where the expression levels of miRNA-122 negatively correlate with the success of IFN therapy (Sarasin-Filipowicz et al, 2009). Hence, performing miRNA profiling in the context of infectious diseases, especially TB, could open a new window for a deeper understanding of pathogenic mechanisms. Furthermore, such signatures represent a promising platform for the development of new diagnostic and prognostic tools.

4.5 miRNA-133b: one miRNA, many targets

Currently, an important question in the miRNA field is whether these small regulatory molecules achieve their physiological impact through repression of a single or a few cardinal targets, or via the cumulative impact of impairing the expression of large sets (sometimes hundreds) of targets (Wallach & Kovalenko, 2009). Whereas most experimental evidence supports the latter option, there are some reported cases where siRNA-mediated down-regulation of one single gene resembled miRNA-mediated phenotypes. For instance, in mice,

specific knockdown of SHIP1 in the hematopoietic system following retroviral delivery of a miRNA-155-formatted siRNA against SHIP1 resulted in a myeloproliferative disorder, with striking similarities to that observed in miRNA-155-over-expressing mice (O'Connell et al, 2009). According to the data presented here, four critical anti-apoptotic genes could be identified, whose expression is impaired simultaneously (directly or indirectly) by miRNA-133b. Importantly, as determined by gene-specific knockdown experiments using siRNAs, none of these genes was capable by itself of phenocopying the pro-apoptotic effect of miRNA-133b in its whole magnitude (data not shown). This supports the idea that the effect mediated by miRNA-133b is the consequence of multiple synergistic small changes in the expression levels of various genes, rather than being attributable to the impaired expression of one single master gene. Nevertheless, it is impossible to exclude that the latter might be the case and, that the identity of the most important miRNA-133b target is still missing. Further studies are necessary in order to increase the list of genes being regulated by miRNA-133b.

4.6 Technical aspects: The challenge of miRNA-target identification

Regardless of the enormous advancement achieved in the field of miRNA research during the last decade, identification of targets genes is still considered a gigantic technical challenge. This represents a major threat for the expansion of our understanding of miRNA function since the latter is ultimately defined by the genes a given miRNA targets and the effects it has on their expression (Mendes et al, 2009). Currently, most miRNA target fishing experiments use *in-silico* sequence analysis and prediction as a starting point. Though helpful, this kind of bioinformatic approach is still prone to high rates of false positive predicted targets. Furthermore, the fact that miRNAs might exert their function either by provoking mRNA cleavage or translation arrest constitutes a further hurdle for efficient target gene identification. Herein results are presented that were obtained after using a mixed miRNA target identification strategy. This consisted of using state-of-the-art methods to assess the impact of miRNA-133b both on the transcriptome and proteome of transfected cells. As a big advantage, the characterization of miRNA-133b as a pro-apoptotic molecule allowed us to narrow the target search to anti-apoptotic and tumor-suppressing molecules. This system proved to be successful as demonstrated by the discovery of FAIM, OPG, GSTP1 and FASN as genes whose expression is impaired by miRNA-133b. Unfortunately,

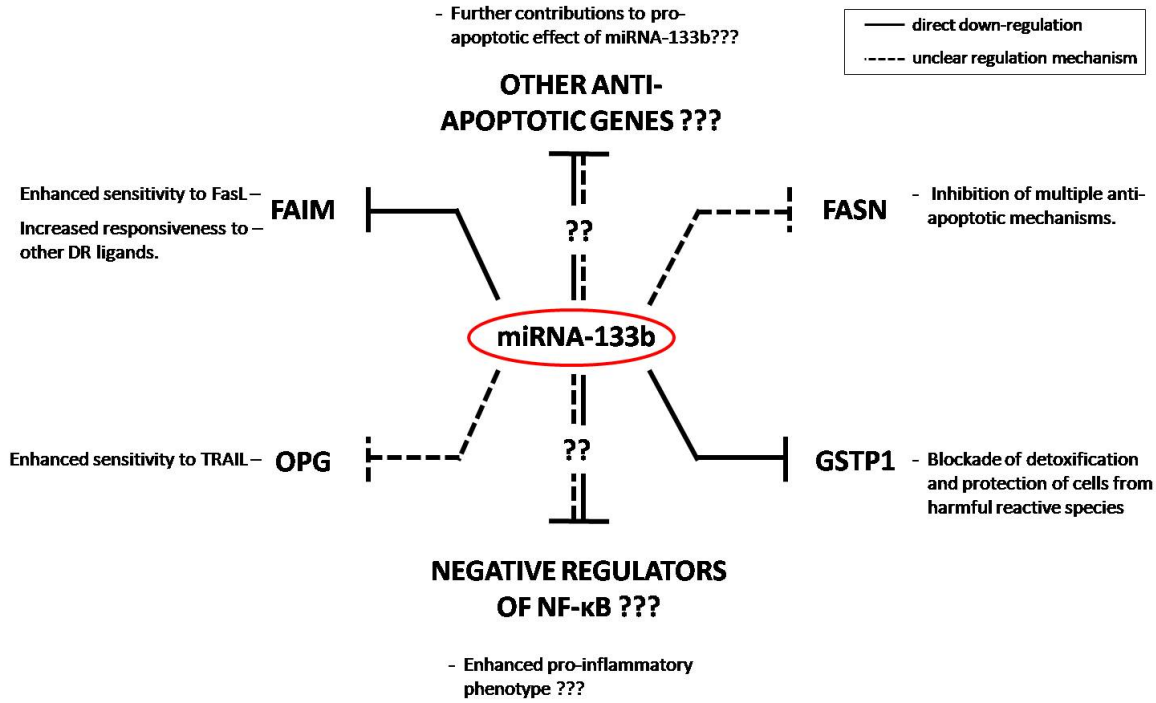
the complexity of this approach as well as the high costs associated to it excludes its application as a routine strategy.

Two more aspects complicate the identification of miRNA targets. First, as discussed previously, miRNA over-expression often leads to rather minute changes of mRNA and protein levels of targeted genes (Baek et al, 2008; Selbach et al, 2008). This raises the question as to what extent small changes in protein levels can be considered biologically relevant. By following the traditional approach of focusing on genes showing the most drastic effects researchers might overlook those that are really important since they take over central roles. MiRNAs exemplify that regulation of biological systems is not a question of strength at the single gene level, but rather of cumulative and synergistic subtle variations of the protein repertoire. Second, as demonstrated by OPG and FASN, finding the exact region being targeted by the miRNA of interest is still not an easy task. It has been recently shown that regions other than the 3´-UTR of a gene can also function as miRNA docking sites (Zhou et al, 2009). Since most of the currently online available *in-vitro* prediction tools do not analyze the entire mRNA sequence, they offer only limited prediction power. Hence, they might not detect and predict numerous non-canonical miRNA binding sites (Lee et al, 2009a; Tay et al, 2008). Moreover, current targeting validation systems are designed to identify binding to the 3´-UTR but not other regions of a gene. Thus, as our knowledge of the mechanisms underlying miRNA function expands, the challenge of designing and establishing more accurate target validation systems is growing.

Last but not least, even if a gene shows an expression pattern that can be specifically influenced by a miRNA (like FASN or OPG in this study), this does not necessarily mean that a direct interaction between the small RNA and mRNA is taking place. Current miRNA target identification techniques do not discern between direct targeting and downstream effects. Hence, in some cases gene regulation might be a consequence of impaired activity of a central gene expression regulator *e.g.* transcription factors. This might be less important for the phenotypic description of miRNA effects, but must be considered when elucidating the exact molecular mechanisms responsible for a given cellular phenotype.

5. Conclusions & Outlook

The work presented here dealt with the characterization of miRNA-133b, a molecule first detected during a screen aimed at the identification of miRNAs differentially regulated during mycobacterial infection of human macrophages. Using an *ex*-vivo model of DR-mediated apoptosis it was shown that miRNA-133b has very strong pro-apoptotic properties. Treatment of HeLa cells with this molecule resulted in strong sensitization towards TNFα or FasL-triggered apoptosis and exacerbated TRAIL sensitivity. Hitherto, this work represents the first description of a miRNA harboring the ability to manipulate the signal pathway composed of DRs and their ligands. As shown by our results, four different mechanisms could be identified by which miRNA-133b renders cells more prone to undergo apoptosis (Scheme 14).



Scheme 14. miRNA-133b as regulator of apoptosis and the inflammatory response mediated by NF-kB

First, it down-regulates the abundance of FAIM and OPG, two anti-apoptotic proteins involved in the regulation of DR-induced cell death in many different biological settings. Secondly, miRNA-133b down-regulates the expression of the oncogenes GSTP1 and FASN. By doing so it impairs the ability of tumor cells to cope with chemical stress and self-supply with important molecules, respectively. Both GSTP1 and FASN have been well characterized in the context of tumorigenesis and immune evasion and represent important therapeutic

targets. Importantly, miRNA-133b represents the first described post-transcriptional expression regulator for all four genes. Hence, our results contribute to a better understanding of the mechanisms governing the expression of central oncogenes. Future work should focus on the characterization of miRNA-133b in different cancer models and its potential as novel target for therapeutic treatment. Given the consistent down-regulation of miRNA-133b in human tumors of different etiology, it would also be interesting to explore its potential application as diagnostic and prognostic marker.

The molecules identified in this work as miRNA-133b targets accomplish important tasks in a variety of biological systems. Furthermore, most of them have been well characterized in the context of disease and represent important therapeutic targets. Given the current knowledge that supports a wider expression pattern of miRNA-133b future work should explore its role during the regulation of processes such as neuronal development, osteogenesis, cardiac and skeletal muscle development, B-cell development or lipogenesis. Further attempts should also aim at expanding and enriching the list of genes targeted by miRNA-133b. This will allow unveiling its biological relevance and regulatory potential.

Herein first evidence was provided for a role of miRNA-133b in the context of innate immunity. Our results link miRNA-133b expression to TLR-mediated pathogen recognition. Notably, contrary to most miRNAs associated so far with innate immunity, miRNA-133b seems to facilitate pro-inflammatory processes rather than to impair them. As demonstrated by our results, miRNA-133b transfection of cells leads to enhanced NF-κB activity and increased synthesis of pro-inflammatory cytokines such as IL6 and 8. Given the central role of NF-κB among transcription factors and its extreme importance for proper biological function, follow-up experiments should definitely aim at elucidating the mechanisms responsible for miRNA-133b mediated enhanced NF-κB activity. In the same line, generation of miRNA-133b and αmiRNA-133b over-expressing macrophages will allow further characterization of the relevance of miRNA-133b in the context of primary immune response to mycobacterial infection.

The miRNA profile presented here is a simplified first attempt to characterize the role of these small regulators during mycobacterial infection. It proves that bacterial challenge modulates miRNA expression levels. Based on this first line of evidence, the possibility of

generating miRNA expression profiles of TB patients should be considered. Currently, the access to more modern sequencing and profiling techniques would allow a deeper and, probably, more sensitive quantification of changes in miRNA levels. This information is very valuable since it could provide new mechanistic insights and, it could have diagnostic and prognostic significance.

Finally, this work constitutes the first characterization of miRNA-133b in the context of DR-mediated apoptosis and innate immunity regulation. Our results show miRNA-133b as a very versatile and potent controller of cellular fate. The evidence provided here, together with the current knowledge about miRNA-133b expression in different cancer types and its role as regulator of the intrinsic apoptotic pathway, indicate that miRNA-133b plays important roles during processes such as cellular transformation and tumor development. Hence, miRNA-133b could represent an interesting molecular target for therapeutic treatment. Follow-up studies should focus on the characterization in other relevant biological models of the molecular mechanisms described here.

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7. Appendix

7.1 Published cancer miRNA expression profiles showing miRNA-133 down-regulation

Table 11. List of published reports showing an impaired expression of miRNA-133b in tumor cells and/or clinical samples

Cancer type	Description	Reference
Multiple cancer types	miRNA-133b specific down-regulation in human biopsies from 8 different tumor types compared to matched healthy tissue	(Navon et al, 2009)
Human non-small cell lung cancer (NSCLC)	miRNA-133b expression is strongly impaired in lung tumor compared to uninvolved tissue	(Crawford et al, 2009)
Head and neck/oral cancer (HNOC)	Several studies demonstrating a lower expression of miRNA-133b in HNOC	(Kozaki et al, 2008; Liu et al, 2009b; Tran et al, 2007; Wong et al, 2008a; Wong et al, 2008b)
Bladder cancer	Impaired expression of miRNA-133b in bladder cancer biopsies compared to healthy control tissue	(Ichimi et al, 2009)
Colorectal cancer	Real-Time PCR examination of colorectal cancer samples and non-neoplastic tissue from patients revealed a diminished miRNA-133b expression	(Bandres et al, 2006)
Human germ cell tumor	Decreased expression of miRNA-133b in testicular germ cell tumors compared to control tissue	(Gillis et al, 2007)

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7.4 Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere, dass diese Arbeit in dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde. Der Inhalt der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt Universität zu Berlin vom 1.09.2005 ist mir bekannt.

Juan P. Patrón
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